THE BOARD OF PATENT APPEALS AND INTERFERENCE IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Man *et al.*	Confirmation No.:	7683
Serial No.:	10/685,942	Art Unit:	1614
Filed:	October 14, 2003	Examiner:	Rae, C.
For:	Pharmaceutically Active Isoindoline Derivatives	Attorney Docket No: CAM:	9516-205-999 501872-999204

BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Pursuant to the provisions of 35 U.S.C. §134 and 37 C.F.R. 41.37, an appeal is taken herein from the final rejection dated July 9, 2008, which rejects claims 1, 5, and 7-8 of this application. A Notice of Appeal was filed in connection with this application on October 8, 2008 with provision for the appropriate fee, and thus, this Brief on Appeal is timely filed.

Also submitted herewith is the fee under 37 C.F.R. §41.20(b)(2), which will be paid electronically via EFS Web.

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I. REAL PARTY IN INTEREST

The real party in interest is Celgene Corporation, the assignee of record of the above-identified application.

II. RELATED APPEALS AND INTERFERENCES

Appellants and their legal representatives respectfully submit that they are not aware of any appeals, interferences, or judicial proceedings that may be related to, directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1, 5, and 7-8 of this application are under final rejection.

Claims 2-4 and 6 were previously canceled without prejudice in connection with a restriction requirement dated July 7, 2004.

New claims 7-8 were added in Appellants' amendment dated October 31, 2007.

Claims 1, 5, and 7-8 are the subject of this appeal.

IV. STATUS OF AMENDMENTS

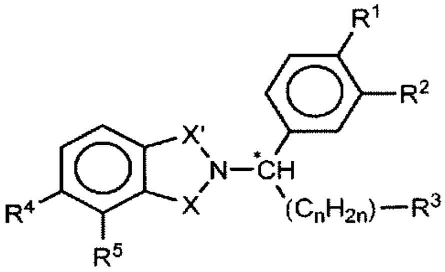
No claim amendments are made in this paper, and thus, no new matter is added.

A listing of claims, showing the claims on appeal, are presented in the **CLAIMS APPENDIX** of this paper.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claims pending in this application generally recite methods of reducing elevated levels of PDE IV in a mammal, or methods of treating an inflammatory or an autoimmune disease in a mammal, which comprise administration of certain chemical compounds. The subject matter recited by each of the pending claims is summarized below.

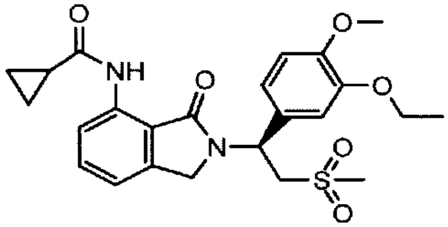
Claim 1 recites a method of reducing elevated levels of PDE IV in a mammal, which comprises administering thereto an effective amount of a compound of the following formula:



or an acid addition salt or a substantially chirally pure isomer thereof, wherein the variables are defined in the claim.

Claim 5 recites a method of treating an inflammatory or an autoimmune disease in a mammal, which comprises administering thereto an effective amount of a compound recited by claim 1.

Claim 7 recites a method of reducing elevated levels of PDE IV in a mammal, which comprises administering thereto an effective amount of a compound of the formula:



or an acid addition salt thereof. The compound recited by claim 7 is within the scope of the genus recited by claim 1.

Claim 8 recites a method of treating an inflammatory or an autoimmune disease in a mammal, which comprises administering thereto an effective amount of a compound recited by claim 7.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues presented on appeal are whether the Examiner's rejection of claims 1, 5, and 7-8 as allegedly not enabled is legally improper in view of *In re Brana*, which is uniquely related to this case and whether the Examiner erred in rejection the claims under the judicially-created doctrine of obviousness-type double patenting over claims 47-70 of U.S. Patent Application No. 10/786,822, particularly in view of the controlling case law, *e.g.*, *Takeda* and *Eisai*.

Specific issues for consideration in this appeal to this Board are:

A. Whether the Examiner erred in rejecting claims 1 and 7 as allegedly not enabled despite the fact that the claims recite methods of reducing an elevated level of PDE IV, and test data showing the efficacy of several representative compounds in reducing the level of PDE IV were indeed provided during the prosecution;

- Whether the rejection of claim 1 is improper where the claim recites a genus of compounds and PDE IV data for several representative compounds encompassed by that genus were provided;
- Whether the rejection of claim 7 is improper where the claim recites a single compound and the data for that single compound was provided;

B. Whether the Examiner erred in rejecting claims 5 and 8 as allegedly not enabled despite the fact that the involvement of PDE IV in inflammatory or autoimmune diseases was known in the art and provided in the specification, and test data showing the efficacy of several representative compounds in reducing PDE IV were submitted during the prosecution;

- Whether the rejection of claim 5 is improper where the claim recites a genus of compounds and PDE IV data for several representative compounds encompassed by that genus were provided;
- Whether the rejection of claim 8 is improper where the claim recites a single compound and the data for that single compound was provided; and

C. Whether the Examiner erred in rejecting claims 1, 5, and 7-8 under the obviousness-type double patenting over claims 47-70 of U.S. Application No. 10/786,822 ("the '822 application")[1]. In particular, whether the allegation made in connection with this rejection that the claims of the '822 application render the current claims "obvious" merely based on alleged "structural similarity," despite the fact that the structures recited by the claims of the '822 application and the pending claims have completely difference structures with regard to position R^3, can be sustained. In addition, whether the inhibition of PDE IV, TNF α, MMP, or undesired angiogenesis, as recited by claims 47-70 of the '822 application, would be patentably indistinct from the treatment of an inflammatory or an autoimmune disease, as recited by the pending claims 5 and 8.

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[1] Appellants respectfully point out that the '822 application issued as U.S. Patent No. 7,345,062, without claims 47-70. However, a continuation application containing claims 47-70 has been filed from the '822 application, and was accorded a serial number 12/009,182.

VII. ARGUMENT

A. Background of the Invention

Many cellular functions are reported to be mediated by levels of adenosine 3',5'-cyclic monophosphate (cAMP). The primary cellular mechanism for the inactivation of cAMP is the breakdown of cAMP by a family of isoenzymes referred to as cyclic nucleotide phosphodiesterases (PDE). (Specification, page 9, citing Beavo *et al.*, *Trends in Pharm.*, 11: 150-155 (1990)). Among the family members of PDE, PDE IV is known to be particularly effective in, among others, inhibition of the inflammatory mediator release. (*Id.*). Thus, reducing PDE IV would "constitute valuable therapeutic strategies for the treatment of many diseases and disorders, including inflammatory and autoimmune diseases. (*Id.*, pages 9-10). The compounds recited by the claims pending in this application are "useful in the inhibition of phosphodiesterases, particularly … PDE IV," and thus, useful "in the treatment of disease states mediated thereby." (*Id.*, page 9).

B. Summary of Prosecution History.

This application was filed on October 14, 2003 as continuation of U.S. Application No. 09/708,199, now U.S. Patent No. 6,667,316 ("the '316 patent), and was accorded a serial number 10/685,942. As originally filed, this application contained 6 claims, directed generally to methods of reducing elevated levels of PDE IV (claim 1), TNFα (claim 2), and matrix metalloproteinase (claim 3) and methods of treating disorders associated with PDE IV (claim 5), TNFα (claim 4), and matrix metalloproteinase (claim 6).

In a restriction requirement issued on July 7, 2004, the claims were restricted to one of three groups: Group I directed to methods of reducing PDE IV and treating disorders associated therewith; Group II directed to methods of reducing TNFα and treating disorders associated therewith; and Group III directed to methods of reducing matrix metalloproteinase and treating disorders associated therewith. In Appellants' response of September 7, 2004, claims in Group I were provisionally selected, and cyclopropyl-N-{2-[1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl]-3-oxoisoindoline-4-yl}caboxamide, the compound (*albeit* with no stereochemical designation) recited by the pending claims 7-8, was elected in connection with the species election requirement.

An Office Action issued on January 10, 2005 by Examiner K. Weddington who no longer is the examiner in the current application. In the office action, claims 1 and 5 were rejected under the non-statutory obviousness-type double patenting over the '316 patent. Despite the fact that the claims of the '316 patent recite compounds and compositions, it was alleged that the methods of use recited by claims 1 and 5 were patentably indistinct from the compounds because the '316 specification "teaches [in] its specification … that the instant compounds inhibit PDE IV, [and] treat inflammatory diseases and/or autoimmune diseases. (January 10 OA, page 3). Additionally, claims 1 and 5 were rejected as allegedly not enabled based on the Examiner's analysis of the factors set forth in *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988) ("the *Wands* factors"). The analysis provided, among others, that the "reducing elevated levels of PDE IV" and "treating an inflammatory or autoimmune disease" are "highly unpredictable and unreliable" (*Id.*, page 4), and that no working examples are provided in the specification. (*Id.*, page 5).

Appellants filed a Response on April 18, 2005. With regard to the rejection under the obviousness-type double patenting, it was pointed out that the rejection is improper because the claims in the '316 patent, which recite composition of matter, are patentably distinct from claims 1 and 5 of the present application, which recite methods of use. (April 18 Response, page 7). Further, it was also pointed out that the Examiner's reliance on the specification of the '316 patent was legally improper. (*Id.*). With regard to the enablement rejection, it was pointed out that: 1) the specification provides sufficient information for those skilled in the art to practice the claimed methods; 2) no objective evidence was provided in the office action that would render the presumption of enablement doubtful; and 3) no undue experimentation is necessary for the practice of the claimed methods. (*Id.*, pages 8-10).

A Final Office Action issued on June 29, 2005, in which Appellants' submission regarding obviousness-type double patenting was dismissed based on the allegation that "the method claims of the present application" is "an obvious variation of the [compounds claimed in the '316 patent] since the method claims cannot function without the compounds of" the '316 patent. (June 29 OA, pages 2-3). In addition, the rejection based on the enable requirement was maintained based on the allegation that "[a]bsent reasonable a priori expectation of success, one skilled in the art would have to test extensively many conditions that may cause elevated PDE levels, many conditions that may cause an inflammatory disease, and … many conditions that may cause an autoimmune disease." (*Id.*, page 5).

Appellants, in their Response dated September 27, 2005, again pointed out that the rejection under the obviousness-type double patenting was legally improper because neither the specification of the '316 patent nor Appellants' own disclosure in the current application may be used to form a basis of the rejection. (September 27 Response, page 2). Further, in connection with the enablement rejection, Appellants pointed out that compliance with the enablement rejection does not turn on whether an example is disclosed. (*Id.*, page 3). Moreover, Appellants provided test results summarizing IC_{50} values for PDE IV in connection with certain exemplary compounds encompassed by the genus recited by claims 1 and 5, and pointed out that, to the extent that there was any doubt with regard to the enablement of the claimed methods, the test results showed that the claimed methods were indeed enabled. (*Id.*, pages 4-5).

In an Advisory Action issued on November 2, 2005, the rejection under the obviousness-type double patenting was withdrawn, but the rejection based on the enablement requirement was maintained.

Appellants filed a Request for Continued Examination and another Response on January 19, 2006. In their Response, Appellants reiterated the fact that the specification provides sufficient guidance to those skilled in the art to practice the claimed methods and resubmitted the PDE IV inhibition data, which was originally submitted in their previous response. (January 19 Response, pages 2-4). In addition, based on the legal principles set forth in *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995), Appellants pointed out that the fact that further research and development are required cannot be the basis for rejecting a claim as not enabled because the *Brana* court recognized the necessity of further research and development particularly in connection with pharmaceutical inventions and held that FDA approval cannot be a prerequisite for patentability. (*Id.*, pages 4-5).

In a subsequent Office Action issued on April 24, 2006, the Examiner reinstated the rejection under the obviousness-type double patenting providing reasons substantially identical to those set forth in previous office actions. (April 24 OA, pages 2-3). Further, also providing reasons substantially identical to those provided in previous office actions, the rejection based on the enablement requirement was also maintained. (*Id.*, pages 3-5). Notably, no reference or comment was made in connection with the test data submitted in Appellants' responses.

In a Response dated June 28, 2006, Appellants again pointed out that the obviousness-type double patenting was legally improper due to the Examiner's improper reliance on the specification of the '316 patent in rejecting the claims. (June 28 Response, pages 2-3). In connection with the enablement rejection, Appellants: 1) resubmitted the test results of certain exemplary compounds and requested that the results be considered; 2) provided a more detailed discussion of the *Brana* case and illustrated that the rejection was improper in view of the legal principles set forth therein; and 3) the specification provides sufficient guidance to those skilled in the art. (*Id.*, pages 4-11).

Another Office Action issued on January 24, 2007 by Examiner C. Rae who is currently the Examiner of this application. With regard to the rejection under the obviousness-type double patenting, it was alleged that the reliance on the '316 specification is proper because "the specification is being used only as a 'dictionary' to determine the meaning of the claim limitations." (January 24 OA, pages 3-4) (emphasis in original). In particular, it is alleged that the specification was referred to to interpret the meaning of "undesirable angiogenesis," as recited by claim 14 of the '316 patent, and it was concluded that the term "undesired angiogenesis," when considered in connection with the '316 patent, would somehow encompass the reduction of PDE IV and the treatment of an inflammatory or an autoimmune disease, as recited by claims 1 and 5 of the present application. (Id., pages 4-5).

With regard to the enablement rejection, it was alleged that there was "doubts ... regarding extrapolation of an [in] vitro method for evaluating the PDE IV inhibitory effects ... to an in vivo method of treating an inflammatory disease or an autoimmune disease." (*Id.*, page 6). In particular, citing Bielekova *et al.*, *The Journal of Immunology*, 164: 1117-1124 (2000) ("Bielekova") and relying on the portion of Bielekova wherein it is purportedly disclosed that "the simple extrapolation of therapeutic efficacy from animal models to human disorders is not easily feasible," the Examiner alleged that there was "reason to doubt" the enablement of claims 1 and 5. (*Id.*, pages 8-10).

In their Response dated April 24, 2007, Appellants pointed out that the obviousness-type double patenting is still improper because the term "undesired angiogenesis," even when read in connection with the '316 specification, does not encompass the reduction of PDE IV or the treatment of an inflammatory or an autoimmune disease because each of these limitations are clearly disclosed as separate embodiments in the '316 specification. (April 24 Response, page 2). Further, it was pointed out that the portions of the '316 specification relied on by the Examiner, even assuming such reliance is legally proper, do not render the reduction of PDE IV or the treatment of an inflammatory or an autoimmune disease obvious so as to make them patentably indistinct from the method recited by claim 14 of the '316 patent. (*Id.*, page 3).

With regard to the enablement rejection, Appellants pointed out that the presumption of enablement was not rebutted by the office action because no proper "reason to doubt" was provided in the office action. (*Id.*, pages 4-5). In particular, it was pointed out that the *Brana* case considered facts similar to those presented in this application and held that the enablement requirement is satisfied even if a reference discloses that the test provided in a patent application may not be indicative of the therapeutic value in humans. (*Id.*). Specifically, Appellants pointed out that the Brana court clearly stated that the test compounds' "ultimate value in humans" cannot be required in assessing patentability. (*Id.*).

An Advisory Action issued on May 18, 2007, simply dismissing Appellants' previous submission as unpersuasive.

Appellants filed a second Request for Continued Examination and a Response on July 20, 2007. The substance of the July 20 response was substantially similar to that of Appellants' response dated April 24, 2007.

Another Final Office Action issued on September 10, 2007, in which the same reasoning with regard to the obviousness-type double patenting and the enablement requirement. Notably, with regard to the obviousness-type double patenting, the Examiner dismissed Appellants' previous submission simply by asserting, without really addressing the substance of Appellants' submission, that the use of the '316 specification was proper because the use was "limited only to its use as a 'dictionary'." (September 10 OA, page 3). With regard to the enablement rejection, the Examiner found Appellants' submission unpersuasive, alleging that the facts of the *Brana* case are distinguishable from those presented in this application. (*Id.*, pages 5-6).

Thereafter, the issues were discussed in an in-person interview held on October 9, 2007. As summarized in "Applicants' Statement of the Substance of the Interview," which was provided in Appellants' Response dated October 31, 2007[2], it was agreed during the meeting that, to the extent that the PDE IV inhibition properties of certain exemplary compounds had been provided during the prosecution, claim 1, which recites, in part, the reduction of PDE IV, would be allowable. Further, with regard to claim 5, which recites the treatment of an inflammatory or an autoimmune disease, it was agreed that Appellants would provide certain references that show that the modulation of PDE IV is implicated with the treatment of various inflammatory or autoimmune diseases. Such references were indeed provided in Appellants' response of October 9. (October 9 Response, page 8). In connection with the rejection under the obviousness-type double patenting, a terminal disclaimer over the '316 patent was filed to expedite the prosecution. (*Id.*, page 7). Lastly, claims 7 and 8, both of which recite, in part, a specific compound encompassed by the genus recited by claims 1 and 5, were added in Appellants' response.

In a Non-final Action issued on December 28, 2007, the rejection under the obviousness-type double patenting was withdrawn in response to the previously filed terminal disclaimer. The rejection based on the enablement requirement, however, was maintained. The reasons provided were: 1) compounds and diseases recited by the claims are very broad; 2) "the term 'method for reducing elevated levels of PDE IV in mammal' ... does not require the presence [of] any pathologic condition"; and 3) the legal principles set forth in *Brana* are inapplicable to this application. (December 28 OA, pages 4-6).

Notably, a new rejection under the non-statutory doctrine of obviousness-type double patent was raised in the office action. Specifically, the claims were rejected as allegedly patentably indistinct from claims 47-70 of the '822 application.

In a Response filed on March 25, 2008, Appellants first pointed out in connection with the enablement rejection that, at the very least, claims 1 and 7 should be patentable regardless of the reasoning provided by the Examiner in the previous office action because claims 1 and 7 recite a specific compound. (March 25 Response, page 2). Further, in connection with the rejection of claims 5 and 8, Appellants reiterated that: 1) the legal principles set forth in *Brana* are indeed applicable to this application; and 2) no proper reason to doubt was provided by the Examiner. (*Id.*, pages 3-6). In connection with the rejection under the obviousness-type double patenting, Appellants pointed out that the claims pending in this application are clearly patentably distinct from those in the '822 application, at least because the claims of the '822 application recite compounds that have a substantially different structure with regard to R^3 position of the currently recited compounds. (*Id.*, page 7).

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[2] The response was filed along with a third Request for Continued Examination.

Despite the response, a Final Office Action issued on July 9, 2008 ("the Office Action"), essentially reiterating the same reasoning provided in previous office actions. This appeal followed.

C. The Examiner Erred in Rejecting Claims 1, 5, and 7-8 Under 35 U.S.C. § 112, ¶1

Appellants respectfully submit that the Examiner erred in rejecting the subject matter recited in instant claims 1, 5, and 7-8 as allegedly not enabled. As the prosecution currently stands, the rejection is premised on the allegation that undue experimentation is required based on the following analysis of the *Wands* factors:

1) Bielekova allegedly provides "reason to doubt" the enablement by illustrating "unpredictability or uncertainty in the art" (Office Action, pages 8-10);

2) since the working examples provided are "limited to in vitro experiments," "specific direction or guidance only for an in vitro methodology for evaluating PDE IV inhibitory effects" was provided (*Id.*, page 11) ;

3) claims 1 and 5 are allegedly very broad as to encompass "multiple variants of the elected compound" and "various diverse conditions involving unregulated angiogenesis" (*Id.*);

4) although claims 7 and 8 recite a single compound, "how the chemical feature of said compound relates to the multiplicity of immunopathologies encompassed by these claims are unclear" (*Id.*); and

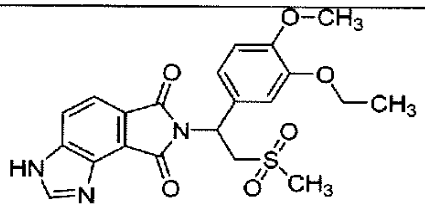
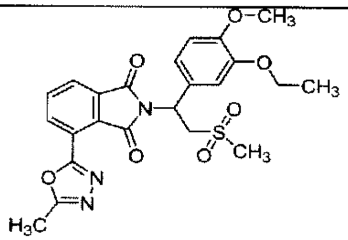
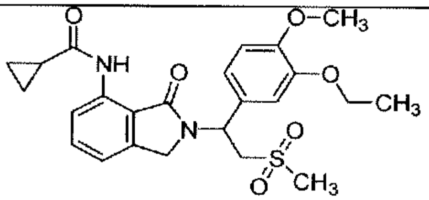
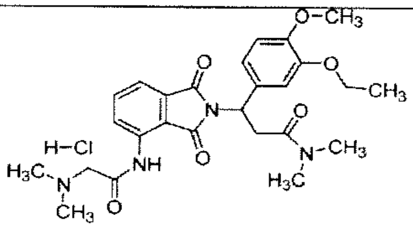
5) the claimed methods of treatment "may differ in terms of method of administration, dose level, [and] dosage regimen," and thus, "coupled with the complex and different immunopathologies associated with inflammatory and autoimmune diseases, ... it is reasonable to surmise that this level of uncertainty and unpredictability in the art would require" more than routine experimentation. (*Id.*, page 12).

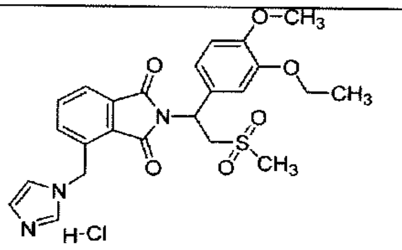
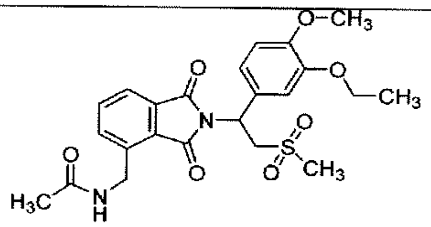
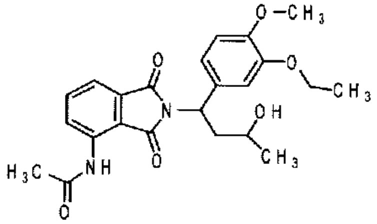
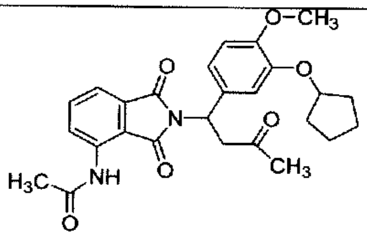
Appellants respectfully disagree with each of these allegations, and submit that no undue experimentation is required to practice the claimed methods for the following reasons.

As well settled, the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. (*U.S. v. Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988)). The Examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. (*In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993)). Furthermore, "[a] specification disclosure...must be taken as being in compliance with the enablement requirement...unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." (Manual of Patent Examining Procedure ("MPEP") § 2164.04) (emphasis added). Moreover, "not everything necessary to practice the invention need be disclosed. All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art." (*Id.*, § 2164.08) (emphasis added).

At the outset, Appellants respectfully submit that the claims pending in this application are clearly enabled because the specification "contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented." (*Id.*, § 2164.04). For example, the specification teaches that PDE IV can be inhibited using compounds of the invention. (Specification, page 24, lines 10-11). Likewise, the specification teaches that an inflammatory or autoimmune disease can be treated by using compounds of the invention. (*Id.*, lines 14-15). Methods of preparing the compounds are described in detail on pages 13-17 of the specification, as well as in the Examples section of this application. Dosages and routes of administration are described, for example, on pages 23-24 of the specification. All that is required for those of ordinary skill in the art to practice the claimed invention is to administer the specified amount of the specified compound using the specified routes of administration. Therefore, it is clear that the application provides sufficient guidance to allow those of ordinary skill in the art to make and use the claimed invention.

Furthermore, for avoidance of any doubt, Appellants provided in their responses *in vitro* data showing the efficacy of certain exemplary compounds in reducing the PDE IV. (*See, e.g.*, Appellants' responses dated January 19, 2006 and September 27, 2005). The data provided during the prosecution are reproduced below:

Example #	Structure	PDE 4 IC_{50} (μM)
3		0.356
15		0.658
55		0.278
61		0.287

65		0.095
66		0.105
71		0.543
76		0.198

The data shown above are further evidence that the compounds recited by the pending claims are indeed effective in inhibiting PDE IV, and thus, the claimed methods are indeed enabled.

1. No proper "reason to doubt" the enablement is provided

Despite the above, the Examiner alleges that the presumption of enablement is rebutted in this application based on the assertion that "there is reason to doubt the objective truth of the statements contained" in this application. (*Id.*). Bielekova, which is attached hereto as **Exhibit A**, was provided to support this allegation. (*Id.*, page 9-10). In addition, along the same line, the Examiner alleges that the data shown above, due to the fact that they were obtained *in vitro*, provides "specific direction or guidance only for an in vitro methodology for evaluating PDE IV inhibitory effects." (*Id.*, page 11) (emphasis added).

However, while passages from Bielekova are extensively reproduced in the Office Action, no explanation as to why the reproduced passages are relevant in determining whether there was a reason to doubt that the claims are enabled. Instead, the only suggestion that Appellants can glimpse from the Office Action is the statement from Bielekova that "simple extrapolation of therapeutic efficacy from animal models to human disorders is not easily feasible." (Office Action, page 10). Presumably, Appellants believe that it is the Examiner's position that there was reason to doubt the enablement because Bielekova states that therapeutic efficacy obtained from animal (or any other models, including the *in vitro* tests provided in this application) cannot easily be extrapolated to human disorders.

Regardless of the truth of that particular statement, Appellants respectfully point out that it is legally erroneous to rely on such a statement in rejecting the claims for allegedly failing to satisfy the enablement requirement. This is because the court in *Brana*, a copy of which is attached hereto as **Exhibit B**, considered a substantially identical disclosure made in a prior art reference (*i.e.*, disclosure in a prior art reference that some laboratory oncologists are skeptical about the predictive value of murine tumor models provided by the applicants for human therapy), and concluded that the claims were enabled because the "usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development." (*Brana*, 51 F.3d at 1568). In reaching this conclusion, the court held that the references that "merely discuss the therapeutic predictive value of [the tests provided by the applicants]" are relevant in assessing the enablement requirement "only if applicants must prove the ultimate value in humans of their asserted utility," but held that no such requirement exists for the patentability. (*Id.* at 1566) (emphasis added).

Applying the holdings of *Brana* to the current application, Bielekova, especially the portion of Bielekova relied on by the Examiner, precisely discloses what the prior art references at issue in *Brana* disclosed (*i.e.*, that the predictive value of certain non-human tests in human therapy may be questionable). However, as the *Brana* court plainly puts it, such a disclosure is relevant "only if applicants must prove the ultimate value in humans of their asserted utility," indicating no requirement to prove the ultimate value in humans exists in connection with patentability. (*Id.*). This is because, according to the *Brana* court, "[t]he stage at which an invention in [the pharmaceutical] field becomes useful is well before it is ready to be administered to humans." (*Id.* at 1568).

Furthermore, Appellants respectfully submit that the Examiner's allegation that "specific direction or guidance only for an in vitro methodology for evaluating PDE IV inhibitory effects" was provided also cannot stand in view of the legal principles set forth in *Brana* discussed above. Indeed, adopting the Examiner's rationale, no claims to method of human treatment can be issued based on *in vitro* data, and such a result would be flatly contrary to Brana court's reasoning that "[t]he stage at which an invention in [the pharmaceutical] field becomes useful is well before it is ready to be administered to humans." (*Brana*, 51 F.3d at 1568)[3].

In this regard, the Examiner maintains that the legal principles set forth in *Brana* are inapplicable to this application based on the assertion that: 1) while the issue in *Brana* was "whether 'asserted utility' was a proper test fro determining enablement under 112, first paragraph," no such issue is present in the current application (December 27 OA, page 5); and 2) the facts of *Brana* are distinguishable from those in the current application since *Brana*'s determination of anti-cancer activity was obtained using "a well-recognized mouse model," whereas "no *in vitro* model is well-recognized in the art for reliably and predictably determining the anti-PDE IV activity … against a specific disease species or the genus of inflammatory and autoimmune diseases." (*Id.*). Appellants respectfully submit that the Examiner is misinterpreting the *Brana* case.

With regard to the Examiner's first allegation that the legal issue presented in *Brana* does not exist in the current application, Applicants respectfully wishes to point to page 1564 of *Brana* decision. There, the court clearly indicated that, although the rejection at issue in *Brana* "appears to be based on the issue of whether the compounds had a practical utility, … the rejection … stands on the requirements of § 112, ¶ 1." (*Brana*, 51 F.3d at 1564) (emphasis added). Thus, the court stated that "it is to that provision that [the court] address" the issue. (*Id.*). In other words, the issue that the court considered and addressed in *Brana* was whether the specification had an enabling disclosure as to the asserted utility of treatment of cancer, an issue substantially identical to the one in this application, *i.e.*, whether the specification had an enabling disclosure as to the claimed utility of the treatment of inflammatory or autoimmune diseases.

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[3] Appellants respectfully point out that there are PDE IV inhibitors approved by the FDA, *e.g.*, Ariflo®. Although FDA approval is not required for patentability, the fact that there are approved PDE IV inhibitors evidences the fact that PDE IV is a valid target for drug development.

In addition, the Examiner also alleges that facts in the current application are distinguishable from those in *Brana* because, according to the Examiner, unlike *Brana*, no well-recognized model for reliably and predictably determining the efficacy for inflammatory and autoimmune diseases is available in the art. (December 27 OA, page 5). First, Appellants respectfully point out that, contrary to the Examiner's allegation, the murine tumor model provided in the application at issue in *Brana* was not a "well-recognized model for reliably and predictably determining the efficacy" of the test compounds. In fact, issues in *Brana* arose precisely because the reliability and predictability of that model was challenged by the examiner based on a prior art reference. Thus, Appellants respectfully submit that the allegation made by the Examiner in this regard is factually incorrect, and thus, cannot be a basis to distinguish the facts of *Brana* from those of the current application.

Furthermore, Appellants respectfully point out that, to the extent that the challenged murine tumor model was found acceptable in *Brana*, the PDE IV test provided by Appellants during the prosecution is sufficient to establish the enablement of the claimed methods. In this regard, Appellants submitted in their response of October 31, 2007 the following references, which show that PDE IV is implicated with various inflammatory and/or autoimmune diseases: 1) Burnouf *et al.*, *Curr. Pharm. Des.*, 8(14): 1255-96 (2002), attached hereto as **Exhibit C**, which indicates that the PDE IV activity is associated with a wide variety of diseases related to inflammatory state or autoimmune pathology (*see*, *e.g.*, Abstract); 2) Dastidar *et al.*, *Curr. Opin. Investig. Drugs*, 8(5): 364-72 (2007), attached hereto as **Exhibit D**, which indicates that PDE IV inhibitors' therapeutic potential in the treatment of various inflammatory disease such as asthma, COPD, psoriasis, rheumatoid arthritis, and inflammatory bowel diseases, *e.g.*, Crohn's disease and ulcerative colitis (*see*, *e.g.*, Abstract); and 3) Sommer *et al.*, *J. Neuroimmunol.*, 79(1): 54-61 (1997), attached hereto as **Exhibit E**, which indicates that PDE IV inhibition is advantageous for the treatment of autoimmune diseases such as multiple sclerosis (*see*, *e.g.*, Abstract). Appellants respectfully submit that these references clearly demonstrate the therapeutic potential of PDE IV inhibition in a wide variety of autoimmune or inflammatory diseases, and thus, the predictive value of the test results provided by Appellants is sufficiently established by these references.

Consequently, Appellants respectfully submit that the legal principles set forth in *Brana* are entirely applicable to this application, and thus, the alleged "reason to doubt" provided by the Examiner is legally improper.

2. The whole scope of the pending claims are adequately enabled

It is alleged in the Office Action that "the claims are very broad," presumably indicating that the whole scope of the pending claims is not enabled. (Office Action, page 11). In this regard, it is alleged that "claim 1 encompasses that all mammalian species, multiple variants of the elected compound, ... various diverse conditions involving unregulated angiogenesis[4], including a heterogen[e]ous group of inflammatory and autoimmune diseases that are characterized by different immunopathologies and etiologies[5]." (*Id.*). Appellants respectfully submit that none of these allegations with regard to claim 1 can form the basis to question the full enablement of claim 1.

First, although the scope of claim 1 does encompass all mammalian species and multiple variants of the elected compound, Appellants respectfully submit that all mammalian species and multiple variants are adequately enabled by the specification and evidence submitted by Appellants during the prosecution. For example, Appellants respectfully submit that the enablement for the genus recited by claim 1 is sufficiently provided in view of the PDE IV inhibition test results provided by Appellants for various compounds encompassed by the genus. While the Examiner appears to imply, without providing any evidence or reasoning, that the whole scope of the genus recited by claim 1 is not enabled, no explanation as to why the PDE IV test data provided for various representative compounds are not sufficient to provide enablement for the genus recited by claim 1. In addition, with regard to the implication that the no sufficient enablement for "all mammalian species" was provided, Appellants respectfully point out that the burden of showing that not all mammalian species were enabled lies on the Examiner. As the Examiner fails to provide any evidence or reasoning to support this implication, Appellants respectfully submit that this burden is not met by the Examiner.

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[4] It is unclear why the Examiner refers to "unregulated angiogenesis" in connection with this analysis. Appellants respectfully point out that "unregulated angiogenesis" is not recited by claim 1.

[5] Presumably, in this part of the statement, the Examiner is referring to claim 5, which recites the treatment of an inflammatory or an autoimmune disease.

Furthermore, while the Examiner alleges that claim 1 encompasses "various diverse conditions involving unregulated angiogenesis," Appellants respectfully point out that claim 1 recites reduction of PDE IV. It is unclear to Appellants as to what limitations of claim 1 are referred to by the Examiner as encompassing "various diverse conditions." In this regard, Appellants respectfully submit that there can be no question regarding the enablement of the subject matter recited by claim 1 because claim 1 simply recites, in part, the reduction of PDE IV, and the reduction of PDE IV by various exemplary compounds encompassed by the genus recited by claim 1 was provided by Appellants. Consequently, Appellants respectfully submit that the whole scope of claim 1 is adequately enabled, and no evidence or reasoning to rebut this presumption is provided by the Examiner.

Claim 5 is also implied by the Examiner to be not fully enabled, allegedly because claim 5 encompasses "a heterogen[e]ous group of inflammatory and autoimmune diseases that are characterized by different immunopathologies and etiologies." (Office Action, page 11). Again, Appellants respectfully submit that such a breadth cannot be equated with the proposition that claim 5 is not fully enabled, especially in view of the fact that Appellants provided ample evidence to show that PDE IV inhibition is implicated with the treatment of various inflammatory or autoimmune diseases.

With regard to claim 7 and 8, while the Examiner recognizes that these claims recite only a single compound, the Examiner indicates that the claims are still not fully enabled allegedly because "how the chemical feature of [the recited compound] relates to the multiplicity of immunopathologies encompassed by these claims are unclear." (*Id.*). First, Appellants respectfully point out that the Examiner is asserting a requirement that finds no root in any of the known legal principles regarding patentability. In other words, understanding or showing "how the chemical feature of [the claimed compound] relates to" the immunopathologies of the disease the compound purports to treat is completely irrelevant to the patentability of a claim to the treatment of the disease using the compound. By the Examiner's logic, no claims to the treatment of a disease should be allowable unless one completely understands the full mechanism of action of the treatment, which is flatly contrary to the legal principles set forth in the *Brana* case. (*See Brana*, 51 F.3d at 1568).

Consequently, Appellants respectfully submit that the breadth of pending claims, in the absence of any evidence or reasoning to rebut the presumption of the enablement provided by the Examiner, cannot be accorded any weight in assessing whether the whole scope of the claims is enabled.

3. No more than routine experimentation is required to practice the claimed methods

It is alleged in the Office Action that the claimed methods of treatment "may differ in terms of method of administration, dose level, [and] dosage regimen," and thus, "coupled with the complex and different immunopathologies associated with inflammatory and autoimmune diseases, ... it is reasonable to surmise that this level of uncertainty and unpredictability in the art would require" more than routine experimentation. (Office Action, page 12). Appellants disagree.

As discussed above, the specification provides sufficient information and guidance with regard to how to practice the claimed methods. Although the Examiner alleges that some experimentation may be required in connection with "method of administration, dose level, [and] dosage regimen," Appellants respectfully point out that the optimization of such factors is no more than what is routinely practiced by those skilled in the art, for which the specification provides sufficient guidance. (*See*, *e.g.*, specification, pages 23-24). Furthermore, Appellants respectfully submit that, even considering the "complex and different immunopathologies associated with inflammatory and autoimmune diseases," no more than routine experimentation is required by those skilled in the art for the practice of the claimed methods.

In this regard, Appellants respectfully point out that the legal principles set forth in *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988) provide helpful insight as to whether undue experimentation would be required to optimize the factors referred to by the Examiner. In *Wands*, the Court of Appeals for the Federal Circuit held that claims directed to immunoassay methods were enabled, even though in order to practice the claimed invention one would have to screen "hybridomas to determine which ones secrete antibody with desired characteristics." (*Wands*, 858 F.2d at 740). This was because "[p]ractitioners of this art are prepared to screen negative hybridomas in order to find one that makes the desired antibody." (*Id.*).

As in *Wands*, the Examiner here is objecting to what is basically a screening step. Yet here, the screening is not nearly as complex as that in *Wands*, as the claimed invention is directed to the use of specific, readily obtainable compounds, for which routes of administration and dosages are set forth in the specification. Moreover, the determination by a physician as to whether a compound is effective in treating a disease in a given patient is a routine determination that is always made by physicians for every pharmaceutical.

Consequently, Appellants respectfully submit that no more than routine experimentation is necessary for the practice of the claimed methods, particularly in view of the direction and guidance provided in the specification and the skill in the art.

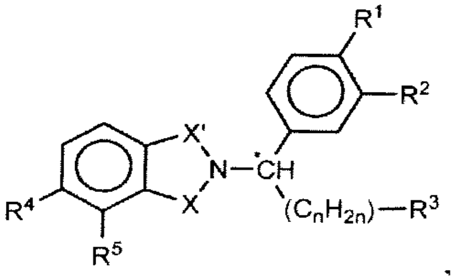
4. Summary

In sum, for the reasons discussed above, the Examiner's rejection is legally improper under *In re Brana*. Further, the analysis of the *Wands* factors set forth by the Examiner, individually or collectively, is simply not sufficient to rebut the presumption of the enablement. Thus, Appellants respectfully request that the Board overturn the Examiner's rejection of the claims under 35 U.S.C. § 112, ¶1.

D. The Examiner Erred in Rejecting the Claims Under the Non-Statutory Doctrine of Obviousness-Type Double Patenting

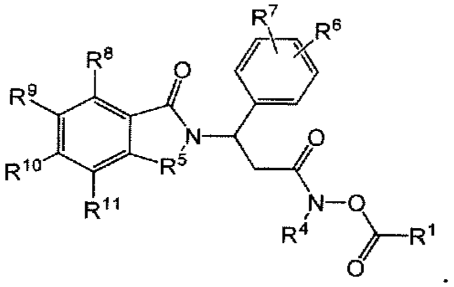
Claims 1, 5, and 7-8 are rejected under the obviousness-type double patenting over claims 47-70 of the '822 application. In particular, it is alleged that the pending claims are not patentably distinct from the claims of the '822 application allegedly because "the instant claims … are deemed to be obvious variants of the referenced claims." (Office Action, page 13).

Claims 1 and 5 of the current application recite, in part, a compound of the following formula:



wherein, among others, R^3 is $-SO_2-Y$, -COZ, -CN, or hydroalkyl of 1 to 6 carbon atoms in which Z is $NR^{6''}R^{7''}$, alkyl of 1 to 6 carbon atoms, phenyl, or benzyl; $R^{6''}$ is hydrogen, alkyl of 1 to 4 carbon atoms, cycloalkyl of 3 to 18 carbon atoms, phenyl, benzyl, or alkanoyl of 2 to 5 carbon atoms, each of which is unsubstituted or substituted with halo, amino, or alkylamino of 1 to 4 carbon atoms; and $R^{7''}$ is alkyl of 1 to 4 carbon atoms.

In contrast, the compound recited by claims 47-70 of the '822 application has the formula:



As can be seen by comparing the two structures, the genus structures recited by claims 47-70 of the '822 application do not even overlap with the genus structure recited by the pending claims. In particular, it is pointed out that the R^3 position of the currently recited genus cannot be $-CO-(NR^4)-O-CO-R^1$ as required by the genus structures recited by claims 47-70 of the '822 application. Therefore, in view of these structural differences, Appellants respectfully submit that the pending claims should be patentably distinct from claims 47-70 of the '822 application.

Despite this, while acknowledging that "the genus structures of the instant claims and the reference claims differ with respect to R3," the Examiner asserts that the pending claims are patentably indistinct from the claims of the '822 application based on the assertion that "it would have been obvious to one of ordinary skill in the art at the time of the invention to select any of the species of the genus taught by the reference, including those of the instant claims, because an ordinary artisan would have reasonable expectation that any of the species of the genus would have similar properties." (Office Action, pages 4-5) (emphasis added).

In this regard, Appellants respectfully point out that the Examiner appears to have misunderstood the chemical structures recited by the pending claims and the claims of the '822 application. To clarify, it is respectfully pointed out that any species that may be selected from the genus recited by the claims of the '822 patent cannot "include those of the instant claims" because the two structures differ on the R^3 position so that there can be no overlap between the structures. Consequently, to the extent that the allegation that the current claims are patentably indistinct from those of the '822 application is based on the Examiner's misunderstanding of the structures recited by the claims, Appellants respectfully submit that the rejection of the claims under the obviousness-type double patenting should be overturned.

In addition, to the extent that the Examiner's rejection may be relying on the allegation of "structural similarity," Appellants respectfully point out that the rejection also cannot stand in view of the controlling legal principles regarding the obviousness of "structurally similar" compounds. (*See, e.g., Takeda Chemical Industries, Ltd. v. Alphapharm Pty., Ltd.* 492 F.3d 1350 (Fed. Cir. 2007), *cert. denied*, 128 S. Ct. 1739 (2008)). Specifically, allegations of "structural similarity" are not enough to render a structural modification obvious because, as the Federal Circuit emphasized, "it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish *prima facie* obviousness of a new claimed compound." (*Id.* at 1357 (emphasis added); *see also Eisai Co., Ltd. v. Dr. Reddy's Laboratories, Ltd.*, No. 2007-1397 (Fed. Cir. Jul. 21, 2008) ("the record contains no reasons a skilled artisan would have considered modification of lansoprazole by removing the lipophilicity-conferring fluorinated substituent as an identifiable, predictable solution.")).

Thus, while the controlling legal principles require that a *prima facie* case of obviousness be supported by "structural similarity" together with a reason to modify, all that is provided in the Office Action is a mere conclusory allegation that the compounds recited by the pending claims are "deemed to be obvious variants of the referenced claims." (Office Action, page 13). As such, no *prima facie* showing that the subject matter claimed by the pending claims is obvious over, and thus, patentably indistinct from, the subject matter recited by the claims of the '822 application is established by the Office Action. For this additional reason, Appellants respectfully request the Board to overturn the rejection under the obviousness-type double patenting.

E. Conclusion

For the reasons discussed above, Appellants respectfully submit that the Examiner erred in rejecting the subject matter of the instant claims. Appellants, therefore, respectfully request that the Examiner's rejections be overturned and that the pending claims be allowed.

A fee of $540.00 is believed due for the submission of this paper, and will be paid via EFS-web. If any additional fees are required, the Director is authorized to charge such fees to Deposit Account No. 50-3013.

Respectfully submitted,

Date: December 8, 2008

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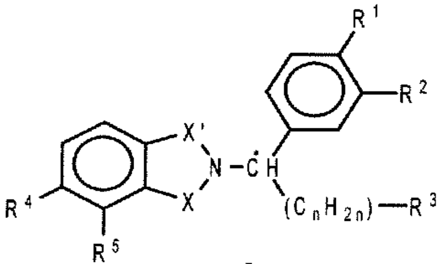
VIII. CLAIMS APPENDIX

1. (Previously presented) A method of reducing elevated levels of PDE IV in a mammal, which comprises administering thereto an effective amount of a compound of formula (I):



I

or an acid addition salt or a substantially chirally pure isomer thereof, wherein:

each of R^1 and R^2, independently of the other, is alkyl of 1 to 4 carbon atoms, alkoxy of 1 to 4 carbon atoms, cyano, cycloalkoxy of 3 to 18 carbon atoms, cycloalkyl of 3 to 18 carbon atoms, or cycloalkylmethoxy in which cycloalkyl has from 3 to 18 carbon atoms;

one of X and X' is =C=O and the other of X and X' is =C=O or $=CH_2$;

R^3 is $-SO_2$-Y, -COZ, -CN, or hydroalkyl of 1 to 6 carbon atoms in which

Y is alkyl of 1 to 6 carbon atoms, phenyl, or benzyl;

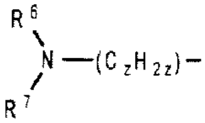
Z is $-NR^{6''}R^{7''}$, alkyl of 1 to 6 carbon atoms, phenyl, or benzyl;

$R^{6''}$ is hydrogen, alkyl of 1 to 4 carbon atoms, cycloalkyl of 3 to 18 carbon atoms, phenyl, benzyl, or alkanoyl of 2 to 5 carbon atoms, each of which is unsubstituted or substituted with halo, amino, or alkylamino of 1 to 4 carbon atoms;

$R^{7''}$ is alkyl of 1 to 4 carbon atoms;

n is 1, 2, or 3;

one of R^4 and R^5 is hydrogen and the other of R^4 and R^5 is imidazolyl, pyrrolyl, oxadiazolyl, triazolyl, or



in which z is 0 or 1;

R^6, when taken independently of R^7, is selected from cycloalkanoyl of 2 to 6 carbon atoms, each of which is unsubstituted or substituted with halo, amino, monoalkylamino or dialkylamino in which each alkyl group contains 1 to 4 carbon atoms; and

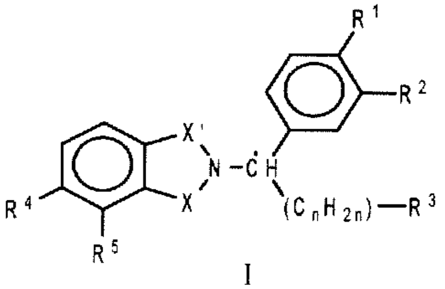
R^7 is hydrogen, alkyl of 1 to 4 carbon atoms, methylsulfonyl, or alkoxyalkylcarbonyl of 2 to 5 carbon atoms; or

R^6 and R^7, taken together, are alkylidene of 1 or 2 carbon atoms substituted by amino, alkylamino, or dialkylamino, in which each alkyl group has from 1 to 4 carbon atoms, or, provided z is 1, -CH=CH-CH=CH-, or -CH=CH-N=CH-; and,

the carbon atom designated * constitutes a center of chirality.

2-4. (Canceled).

5. (Previously presented) A method of treating an inflammatory disease or an autoimmune disease in a mammal, which comprises administering thereto an effective amount of a compound of formula (I):



I

or an acid addition salt or a substantially chirally pure isomer thereof, wherein:

each of R^1 and R^2, independently of the other, is alkyl of 1 to 4 carbon atoms, alkoxy of 1 to 4 carbon atoms, cyano, cycloalkoxy of 3 to 18 carbon atoms, cycloalkyl of 3 to 18 carbon atoms, or cycloalkylmethoxy in which cycloalkyl has from 3 to 18 carbon atoms;

one of X and X' is =C=O and the other of X and X' is =C=O or $=CH_2$;

R^3 is $-SO_2$-Y, -COZ, -CN, or hydroalkyl of 1 to 6 carbon atoms in which

Y is alkyl of 1 to 6 carbon atoms, phenyl, or benzyl;

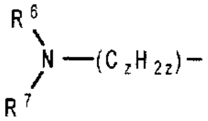
Z is -NR$^{6''}$R$^{7''}$, alkyl of 1 to 6 carbon atoms, phenyl, or benzyl;

R$^{6''}$ is hydrogen, alkyl of 1 to 4 carbon atoms, cycloalkyl of 3 to 18 carbon atoms, phenyl, benzyl, or alkanoyl of 2 to 5 carbon atoms, each of which is unsubstituted or substituted with halo, amino, or alkylamino of 1 to 4 carbon atoms;

R$^{7''}$ is alkyl of 1 to 4 carbon atoms;

n is 1, 2, or 3;

one of R^4 and R^5 is hydrogen and the other of R^4 and R^5 is imidazolyl, pyrrolyl, oxadiazolyl, triazolyl, or



$$\begin{matrix} R^6 \\ & N-(C_zH_{2z})- \\ R^7 \end{matrix}$$

in which z is 0 or 1;

R^6, when taken independently of R^7, is selected from cycloalkanoyl of 2 to 6 carbon atoms, each of which is unsubstituted or substituted with halo, amino, monoalkylamino or dialkylamino in which each alkyl group contains 1 to 4 carbon atoms; and

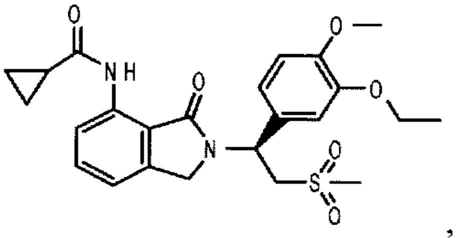
R^7 is hydrogen, alkyl of 1 to 4 carbon atoms, methylsulfonyl, or alkoxyalkylcarbonyl of 2 to 5 carbon atoms; or

R^6 and R^7, taken together, are alkylidene of 1 or 2 carbon atoms substituted by amino, alkylamino, or dialkylamino, in which each alkyl group has from 1 to 4 carbon atoms, or, provided z is 1, -CH=CH-CH=CH-, or -CH=CH-N=CH-; and,

the carbon atom designated * constitutes a center of chirality.

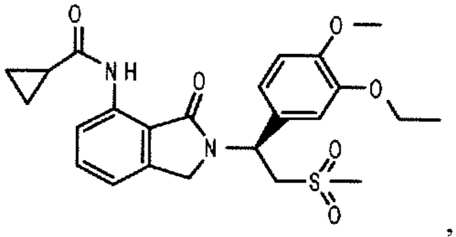
6. (Canceled).

7. (Previously presented) A method of reducing elevated levels of PDE IV in a mammal, which comprises administering thereto an effective amount of a compound of formula:



or an acid addition salt thereof.

8. (Previously presented) A method of treating an inflammatory disease or an autoimmune disease in a mammal, which comprises administering thereto an effective amount of a compound of formula:



or an acid addition salt thereof.

IX. EVIDENCE APPENDIX

Exhibit A: Bielekova *et al., The Journal of Immunology*, 164: 1117-1124 (2000)

Exhibit B: *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995)

Exhibit C: Burnouf *et al., Curr. Pharm. Des.*, 8(14): 1255-1296 (2002)

Exhibit D: Dastidar *et al., Curr. Opin. Investig. Drugs*, 8(5): 364-372 (2007)

Exhibit E: Sommer *et al., J. Neuroimmunol.*, 79(1): 54-61 (1997)

X. RELATED PROCEEDINGS APPENDIX

None.

Exhibit A

Therapeutic Potential of Phosphodiesterase-4 and -3 Inhibitors in Th1-Mediated Autoimmune Diseases

Bibiana Bielekova, Anne Lincoln, Henry McFarland, and Roland Martin[1]

Phosphodiesterase-4 (PDE4) inhibitors have the potential to modulate immune responses from the Th1 toward the Th2 phenotype and are considered candidate therapies for Th1-mediated autoimmune disorders. However, depending on the model and cell types employed, studies of atopic individuals have come to the opposite conclusion, i.e., that PDE inhibitors may be beneficial in asthma. Using in vitro immunopharmacologic techniques we analyzed the effects of PDE4 and PDE3 inhibitors on human immune cells to address these discrepancies and broaden our understanding of their mechanism of action. Our results indicate that PDE inhibitors have complex inhibitory effects within in vivo achievable concentration ranges on Th1-mediated immunity, whereas Th2-mediated responses are mostly unaffected or enhanced. The Th2 skewing of the developing immune response is explained by the effects of PDE inhibitors on several factors contributing to T cell priming: the cytokine milieu; the type of costimulatory signal, i.e., up-regulation of CD86 and down-regulation of CD80; and the Ag avidity. The combination of PDE4 and PDE3 inhibitors expresses synergistic effects and may broaden the therapeutic window. Finally, we observed a differential sensitivity to PDE inhibition in autoreactive vs foreign Ag-specific T cells and cells derived from multiple sclerosis patients vs those derived from healthy donors. This suggests that PDE inhibition weakens the strength of the T cell stimulus and corrects the underlying disease-associated cytokine skew in T cell-mediated autoimmune disorders. These new findings broaden the understanding of the immunomodulatory actions of PDE inhibitors and underscore their promising drug profile for the treatment of autoimmune disorders. ***The Journal of Immunology,* 2000, 164: 1117–1124.**

Hyperactive Th1-mediated immune responses are thought to be involved in the pathogenesis of many autoimmune diseases, including multiple sclerosis (MS).[2] Phosphodiesterases (PDE) are enzymes degrading the second messenger cAMP, which mediates and regulates essential intracellular processes (1). There are 10 different PDE families, but immune cells predominantly express families PDE4, PDE3, and, to a lesser extent, PDE7 (2, 3). Although no PDE7 inhibitor is available, the inhibitors of PDE4 and PDE3 families exert complex immunomodulatory properties. In animals, these drugs inhibit Ag-mediated T cell proliferation and skew the T cell cytokine profile toward a Th2 phenotype by down-regulating the expression or production of Th1 cytokines (4–6) and have no effect or even augment the production of Th2 cytokines (6, 7). These properties render PDE inhibition a candidate therapy for Th1-mediated autoimmune disorders. Indeed, both nonselective as well as PDE4-specific inhibitors were effective in ameliorating disease in different experimental autoimmune encephalomyelitis models (5, 8–10) and in collagen-induced arthritis models (11, 12). However, the simple extrapolation of therapeutic efficacy from animal models to human disorders is not easily feasible (13), and therefore, the analysis of the immunomodulatory properties of PDE inhibitors on human immune cells is an important step in preclinical testing. Studies exploring the effects of PDE inhibitors in humans in vitro or in vivo are still limited. Although there is some evidence for a preferential inhibition of proinflammatory cytokines in Th1-mediated human autoimmune conditions (2, 14, 15), data from asthmatic and atopic individuals reached almost the opposite conclusion, i.e., that these drugs lead to preferential inhibition of Th2 responses (16). We therefore decided to study the effects of the selective PDE4 inhibitor rolipram and the PDE3 inhibitor cilostamide on a large number of human autoreactive and foreign Ag-reactive T cell lines (TCL) derived from MS patients and healthy individuals and asked the following questions. 1) Which biological functions (Ag-specific proliferation, cytokine production, functional Ag avidity) of human CD4$^+$ T cells are influenced by these PDE inhibitors? 2) Can these effects be induced by the concentrations of drugs achievable in vivo? 3) Is there a differential effect between PDE4 and PDE3 inhibitors and their combination? 4) Is there a differential sensitivity to the PDE inhibition between autoreactive and foreign Ag-reactive TCL and between TCL derived from MS patients vs healthy donors? Finally, 5) what are the possible explanations of the immunomodulatory effect of PDE inhibitors?

Materials and Methods

Reagents, generation of T cell lines, and proliferation assays

Rolipram (racemate of 4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone) was provided by Dr. Harald Dinter (Berlex Laboratories, Richmond, CA). Cilostamide (OPC 3689) was a gift from Dr. Vincent Manganiello Pulmonary-Critical Care Medicine Branch (PCCMB), National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). Fresh solutions of individual drugs were prepared for each experiment. The solvent for both drugs, DMSO (Sigma, St. Louis, MO), was used in 1/1000 dilution with T cell medium for the 10-μM concentration of drugs and at this concentration did not influence T cell proliferation when used as a negative control.

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[2] Abbreviations used in this paper: MS, multiple sclerosis; PDE, phosphodiesterase; MBP, myelin basic protein; TCL, T cell line.

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Myelin basic protein was prepared as previously described (17). The peptides Flu-HA$_{306-318}$ (PKYVKQNTLKLAT) and tetanus$_{830-843}$ (QYIKANSKFIGITQL) were synthesized by continuous flow, solid phase peptide synthesis on the basis of the F-moc/Bu^t strategy. Peptides were purified by HPLC, and their identities were tested using ion spray mass spectrometry.

The TCL were generated by an IL-7-modified primary proliferation assay, a method that allows the rapid expansion of Ag-specific T cells, including in vivo activated cells. Briefly, PBMC were isolated from fresh leukophereses by Ficoll density gradients and were seeded in 96-well U-bottom plates in T cell medium (IMDM, Life Technologies, Grand Islands, NY) containing 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin/streptomycin (all from BioWhittaker, Walkersville, MD), and 5% pooled human plasma at 1 × 10^5 cells/well, with addition of 10 ng/ml IL-7 (recombinant human IL-7; PeproTech, Rocky Hill, NJ). After 7 days (37°C and 5% CO_2), cultures were split by transferring 100 μl of each cell culture into a daughter plate, which was pulsed with [^{3}H]thymidine (Amersham, Arlington Heights, IL) at 1 μCi/well. The incorporated radioactivity (counts per minute) was measured by scintillation counting (Betaplate, Pharmacia LKB, Piscataway, NJ) 8 h later. Proliferation of cultures with Ag (25 μg/ml MBP or 5 μg/ml peptides) was compared with proliferation of negative control wells seeded without Ag. Positive cultures (stimulation index >2 and absolute counts per minute at least 3 SDs above the average counts per minute of negative control wells) were identified on the mother plates and were periodically restimulated. The Ag specificity was confirmed at the end of the second in vitro stimulation cycle in 48-h proliferation assays as previously described (18). All blood samples were collected according to an institutional review board-approved protocol, and informed consent was obtained before the study. None of the patients received any immunomodulatory or immunosuppressive treatment within 1 mo before blood collection.

Effects of PDE-4 and PDE-3 inhibitors on Ag-specific TCL proliferation and functional Ag avidity

The effects of PDE inhibitors on Ag-specific proliferation, cytokine production, and functional Ag avidity were assessed during the third stimulation cycle (days 22–25 ex vivo). Each drug was used alone (in three concentrations: 0.1, 1, and 10 μM) or in combination (0.05, 0.5, and 5 μM, concentrations of individual drugs in the mixture). The selected dose range included the concentrations achievable in vivo (for rolipram, 0.09 and 0.2 μM; Dr. Claus-Steffen Stuerzebecher, Schering, Berlin, Germany, personal communication). Each condition was tested in duplicate, including negative (no Ag) and positive (Ag, no drug) controls. T cells were plated in 96-well U-bottom plates at 2 × 10^4 T cells/well with irradiated autologous PBMC at 1 × 10^5 cells/well. The Ag was added at the seeding concentration (25 μg/ml MBP and 5 μg/ml peptides) or over a wide range of Ag concentrations for the functional Ag avidity assay. Supernatants from these assays were collected after 36 h of incubation and stored frozen until analysis. For the last 8 h of incubation, cells were pulsed with [^{3}H]thymidine at 1 μCi/well, and the incorporated radioactivity was measured by scintillation counting.

Cytokine secretion

Secretion of a Th1 (IFN-γ) and a Th2 (IL-4) cytokine by Ag-specific TCL was assessed by sandwich ELISA (Cyto-Sets from BioSource International, Camarillo, CA) according to the manufacturer's recommendation. All standards and samples were tested in duplicate.

Flow cytometry (FACS) analysis of the surface expression of costimulatory molecules

Fresh PBMC (1.2 × 10^6 cells/ml) were seeded in bulk cultures in 48-well plate with or without rolipram (10 μM). In addition to nonstimulated cells, the effect of rolipram was assessed upon nonspecific stimulation with LPS (2.5 μg/ml) or PHA-P (PHA; 5 μg/ml; both from Sigma). After 12-h incubation cells were washed with wash buffer (Dulbecco's PBS with 1% heat-inactivated FCS and 0.1% sodium azide) and incubated with fluorescein-, PE-, or Cy-Chrome-conjugated Ab (HLA-DR,DP,DQ-FITC, CD19-FITC, CD14-FITC and -PE, CD80-FITC and -PE, CD86-FITC and -PE, and CD3-Cy-Chrome; all from PharMingen, San Diego, CA) at saturating concentrations for 30 min on ice, then washed three times and analyzed (FACScan, Becton Dickinson, CA) using Cell-Quest software. Isotype-matched mouse IgG negative controls were used for each staining. Monocytes were gated based on the size characteristics (forward and side scatter), and expression of CD14. Lymphocytes were identified by the size characteristics and differentiation between T and B lymphocytes was based on the expression of CD3 and CD19 molecules, respectively. Five thousand cells in the gated population were analyzed per sample.

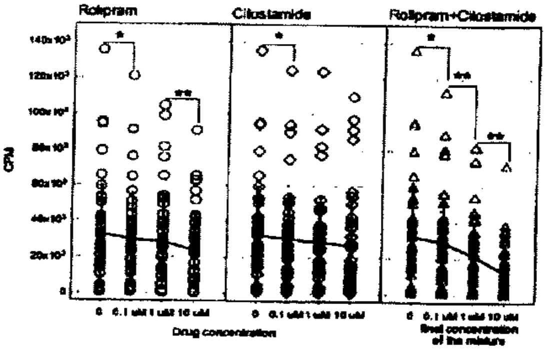

FIGURE 1. Effect of PDE inhibitors on Ag-specific proliferation of TCL. A total of 47 TCL was stimulated with specific Ag without or with three increasing concentrations of rolipram and cilostamide. For the combination of rolipram and cilostamide, each drug was used in concentrations of 0.05, 0.5, and 5 μM, respectively. Results are depicted as counts per minute. Statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$). Only rolipram and the combination of both drugs had a dose-related inhibitory effect on Ag-specific TCL proliferation. The combination of rolipram and cilostamide was the most potent, demonstrating a synergistic effect of individual drugs. The differences among three treatment modalities reached statistical significance ($p < 0.05$, by repeated measures ANOVA analysis).

Statistical analysis

The data were analyzed by a commercial software package (Sigma-Stat, SPSS, Chicago, IL). The effects of the drugs on biological functions of TCL was evaluated by one-way repeated measures ANOVA or, if normality failed, by Friedman's repeated measure analysis on ranks. Statistically significant differences from repeated measures ANOVA were further analyzed by the Student-Newman-Keuls test, with $p < 0.05$ as a cut-off for statistical significance. The effect of rolipram on costimulatory molecules was assessed by the Mann-Whitney rank-sum test.

Results

Effect of PDE-4 and PDE-3 inhibitors on Ag-specific TCL proliferation

We examined the effects of rolipram and cilostamide on the proliferation of 47 Ag-specific TCL. Both drugs inhibited Ag-driven TCL proliferation, but only rolipram and the combination of both drugs expressed statistically significant inhibitory effects in a dose-dependent manner (Fig. 1). The inhibition by rolipram (8.8–32% inhibition) was more pronounced than that by cilostamide (6.31–22.61% inhibition). Combinations of both drugs (1/1 ratio and half molar concentration of each drug (0.05, 0.5, and 5 μM in the final mixture) proved to be most efficient in inhibiting TCL proliferation (10.34–61.05%), exceeding the additive effects of individual drugs. The observed differences between drugs and their combination were statistically significant ($p < 0.05$, by repeated measures ANOVA). Because the above characteristics of the drug combination indicated synergistic effects, we have quantified the degree of synergism between rolipram based on the modified Berenbaum equation (19):

The degree of synergism between drugs A and B is equal to $1/R$, where $R = [(IC_{20}\text{ drug A} + B)/(IC_{20}\text{ drug A})] + [(IC_{20}\text{ drug B} + A)/(IC_{20}\text{ drug B})]$.

Based on the proliferation data in Fig. 1 we have estimated the IC_{20} (the concentration of each drug that leads to 20% inhibition of TCL proliferation; this was substituted for IC_{50}, as neither rolipram nor cilostamide reached 50% of inhibition) from the dose-response curves and calculated $R_{\text{rolipram + cilostamide}} = 0.1123$. The

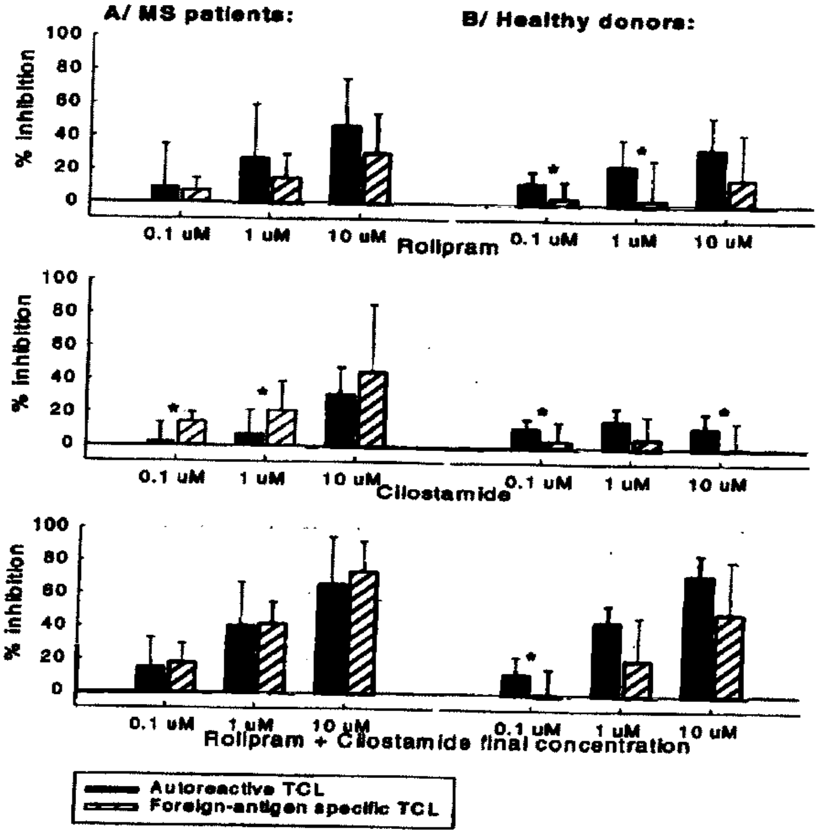

FIGURE 2. Effects of PDE inhibitors on proliferation of TCL stratified based on their origin (derived from MS patients vs healthy donors) and selecting Ag (autoreactive vs foreign-Ag specific). Upon stratification of the proliferation data with respect to the origin of the TCL and the type of selecting Ag, a hierarchy of the sensitivity to PDE inhibition emerged; MS-derived TCL were more sensitive than healthy donor (HD)-derived TCL. A similar differential sensitivity to PDE4 vs PDE3 inhibition was observed in the analysis of autoreactive and foreign Ag-specific TCL within each population group. Statistically significant differences are indicated ($p < 0.05$, by Friedman's repeated measures analysis on ranks).

degree of synergism between rolipram and cilostamide is $1/R = 8.9$. (Values <1 indicate antagonism, 1 indicates additivity, and values >1 indicate synergism.)

Comparison of the susceptibility to PDE-4 and -3 inhibition by autoreactive and foreign Ag-specific TCL and by TCL derived from MS patients and healthy donors

When the inhibitory effect of PDE inhibitors was assessed after stratification of TCL based on their origin and the type of the selecting Ag (TCL derived from MS patients (26 TCL, 17 autoreactive, 9 foreign-Ag-specific) vs. TCL derived from healthy donors (21 TCL, 5 autoreactive, 16 foreign-Ag-specific)), two interesting observations emerged (Fig. 2). 1) The MS-derived TCL were more susceptible to the effect of PDE inhibitors than were the healthy donor-derived TCL ($p < 0.05$ for 10 μM rolipram, 10 μM cilostamide, and 0.1 μM and 1 μM concentrations of drug mixture; Friedman's repeated measures analysis on ranks). 2) Also, there appeared to be a differential sensitivity of autoreactive TCL and foreign Ag-specific TCL to the effects of PDE4 and PDE3 inhibition. Autoreactive TCL were inhibited by rolipram to a greater extent than foreign Ag-specific TCL ($p < 0.05$, through the tested concentration range of rolipram, Friedman's repeated measures analysis on ranks). Only stratified data are depicted in Fig. 2. Due to the decreased power of a comparison of divided data into individual patient groups, not all differences reached statistical significance ($p < 0.05$, by Friedman's repeated measures analysis on ranks); these are marked with an asterisk.

Effect of PDE inhibitors on cytokine production by Ag-specific TCL

We evaluated the effects of PDE inhibitors on Ag-driven production of two cytokines, IFN-γ (prototypic Th1 cytokine) and IL-4 (prototypic Th2 cytokine; Fig. 3). Although a significant variability was noted among individual TCL, the average effect of PDE4 inhibition by rolipram and of PDE4 and PDE3 inhibition by the combination of drugs on IFN-γ production was inhibitory. Rolipram (12.26–27.8% inhibition) and the combination of both drugs (16.07–46.3%) inhibited IFN-γ synthesis in a dose-dependent manner. The effect of cilostamide was mild and did not reach statistical significance for any concentration (4.47–11.27% inhibition). Differences among the drugs were again statistically significant.

Neither rolipram nor the combination of rolipram and cilostamide had a statistically significant effect on IL-4 production (inhibition ranging from −0.62 to 1.69% throughout the concentration range). Cilostamide had a mild inhibitory effect on IL-4 production at 0.1 μM, whereas a high concentration (10 μM) significantly enhanced IL-4 production (−11.39% inhibition at 10 μM concentration). Overall, the effect of PDE4 and PDE3 inhibitors on IFN-γ production paralleled those on proliferation, with the drug combination having synergistic effects. The IL-4 production was largely unaffected despite the demonstrated significant inhibition of TCL proliferation at the drug concentrations tested.

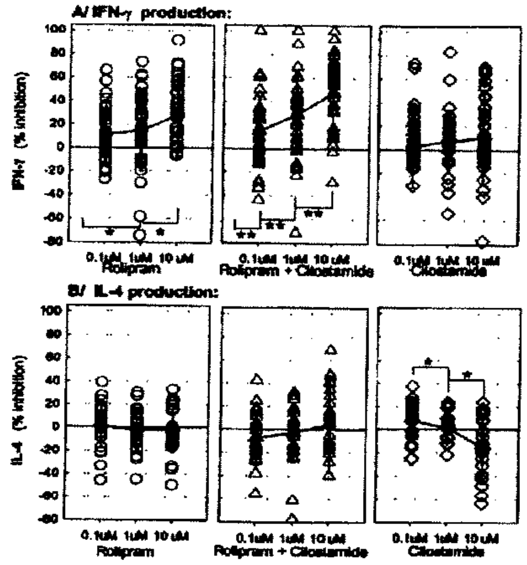

FIGURE 3. Effect of PDE inhibitors on cytokine production of Ag-specific TCL. The effects of rolipram, cilostamide and their combination on Ag-specific production of IFN-γ and IL-4 was assessed in parallel to the proliferation. Results are depicted as the percent inhibition of cytokine production for each concentration of drug compared with control cultures (no drug added). Statistically significant differences are indicated (*, $p < 0.05$; **, $p < 0.01$). Although all TCL tested in proliferation assays produced IFN-γ and are included in the analysis, only 9 of 26 MS-derived TCL and 17 of 21 healthy donor-derived TCL produced IL-4; therefore, only these 26 TCL could be included in the analysis of the effect of PDE inhibitors on IL-4 production. Only rolipram and the combination of rolipram with cilostamide inhibited the production of IFN-γ significantly compared with control samples. This inhibition paralleled the effect on TCL proliferation and was clearly strongest for the combination of both drugs. Neither rolipram nor the drug combination has a significant effect on IL-4 production. A high concentration (10 μM) of cilostamide enhanced IL-4 production despite the demonstrated inhibitory effect on proliferation. Differences between individual drugs were statistically significant ($p < 0.05$).

Effect of PDE inhibitors on the functional Ag avidity of TCL

To gain a better understanding of the mechanism of the PDE4 and PDE3 inhibitor-induced bias of immune responses from a Th1 toward a Th2 phenotype, we decided to study their effects on the major components of T cell priming. Apart from the influence on the cytokine milieu, which was supported by the above experiments, we wanted to address the question of whether these drugs influence the Ag dose needed for T cell activation, i.e., antigen avidity, and costimulatory signals delivered by APC. First, to address the question of whether the magnitude of the immunomodulatory effect of PDE inhibitors on TCL varies depending on the dose of Ag, we exposed a subgroup of TCL (15 TCL; selected as a representative sample based on their phenotype, variable susceptibility to PDE inhibition, and reactivity to either autoantigen or environmental Ag; Table I) to a 1-μM concentration of the individual drugs over a wide range of Ag concentrations. The results of this functional Ag avidity assay for three TCL (of Th1, Th0, and Th2 phenotypes) are summarized in Fig. 4. None of the autoreactive or foreign Ag-specific TCL that we generated for this project expressed a clear Th2 phenotype. We therefore included a Th2-like TCL specific for copolymer-1 (Cop-1), an approved immunomodulatory drug for MS. The TCL Th1:MBP and Th0:Flu-HA were representative of all other tested TCL and illustrate the above-mentioned differential effect of PDE inhibition on autoreactive vs foreign Ag-reactive TCL. All examples also demonstrate the hierarchy of immunomodulation between individual drugs (cilostamide < rolipram < rolipram + cilostamide). For MBP-specific Th1-TCL, a combination of PDE4 and PDE3 inhibition resulted in almost 100% inhibition of proliferation and IFN-γ production. For Flu-HA-specific Th0-TCL, the inhibition of proliferation and IFN-γ production was much less pronounced (~50% with the combination of drugs for each Ag concentration), and the production of IL-4 was either unaffected or even enhanced. For Cop-1-specific Th2-TCL, the individual drugs had no effect on Ag-specific proliferation or IL-4 production. Overall, the magnitude of the effect of PDE inhibitors on individual TCL was similar through the tested Ag concentration range. However, as demonstrated in Table I, the PDE inhibition had a mild, but statistically significant, effect on the EC_{50} (concentration of Ag that leads to 50% maximal proliferation) of individual TCL ($p = 0.009$, Friedman's repeated measures analysis on ranks).

Table I. *Effect of PDE inhibitors on TCL Ag avidity (EC_{50})*

				EC_{50} (μg/ml)			
TCL	Ag	TCL Origin	Phenotype	No drug	Cilostamide	Rolipram	Rolipram + cilostamide
1	Flu-HA	MS	Th1	0.070	0.070	0.080	0.200
2	Flu-HA	MS	Th1	0.070	0.200	0.200	0.200
3	Flu-HA	MS	Th1	0.009	0.010	0.030	0.500
4	Flu-HA	MS	Th0	0.003	0.003	0.100	0.100
5	Flu-HA	MS	Th0	0.008	0.010	0.100	0.100
6	MBP	MS	Th1	0.050	0.070	0.070	0.100
7	MBP	MS	Th1	10.000	10.000	—[a]	—[a]
8	MBP	MS	Th1	2.000	3.000	2.000	3.000
9	MBP	MS	Th1	1.500	1.500	1.500	1.400
10	MBP	HD	Th1	20.000	20.000	20.000	30.000
11	MBP	MS	Th0	3.000	3.000	10.000	—[a]
12	MBP	MS	Th0	1.000	1.500	1.000	1.000
13	MBP	HD	Th0	25.000	25.000	25.000	25.000
14	MBP	HD	Th0	3.000	3.000	4.000	3.000
15	Cop-1	HD	Th2	3.000	3.000	3.000	3.000
Total	Mean ± SD			4.58 ± 7.77	4.69 ± 7.73*	4.79 ± 8.02*	5.20 ± 10.02*

[a] 100% inhibition of proliferation.

*, $p < 0.05$; Friedman's repeated measures analysis on ranks with Student-Newman-Keuls test.

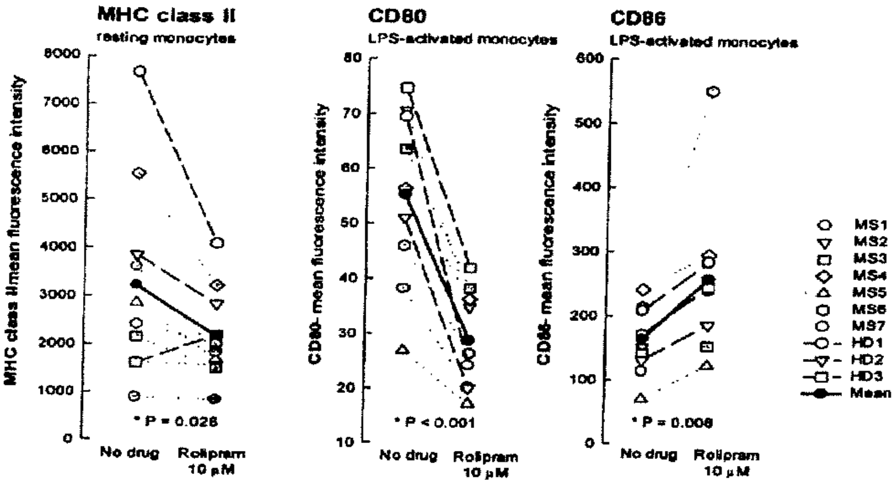

FIGURE 5. Effect of rolipram on the expression of costimulatory molecules by APC. The surface expression of MHC class II and costimulatory molecules CD80 and CD86 on monocytes and B and T lymphocytes was assessed by triple-staining, flow cytometric analysis. Resting or nonspecifically activated (LPS or PHA) PBMC were incubated for 12 h in bulk culture with or without rolipram and analyzed by flow cytometry. Only the effect on monocytes (gated based on size characteristics and CD14 expression), both rested and activated by LPS, is depicted in this figure, but similar results were obtained upon activation by PHA or when gating of B or T lymphocytes. Rolipram down-regulates MHC class II expression on both resting and activated cells, while it down-regulates CD80 and up-regulates CD86 expression upon activation with either nonspecific stimulus. These changes were observed in both MS patients and healthy donors.

patients have biased immune responses toward opposite Th phenotypes as compared with unbiased responses of healthy donors, we asked whether the explanation for these controversial data from human TCL lies in the differential susceptibility to PDE inhibition between these different patient groups. This hypothesis prompted us to examine the effect of PDE inhibition on large numbers of TCL, derived from both healthy donors and MS patients, and with specificity for two Ags, the autoantigen MBP and the classical foreign recall Ags Flu-HA or tetanus (22). Indeed, we were able to demonstrate a higher susceptibility to PDE inhibition by MS-derived compared with healthy donor-derived TCL. This finding has several important implications. It explains how the same therapeutic agent could be considered for the treatment of disorders with potentially different pathogenesis (Th1-mediated autoimmune disorders vs. Th-2-mediated asthma and atopic dermatitis). We and others recently demonstrated (23, 48) that the immune system in MS patients is in a dysregulated state characterized by an overshooting Th1 response not only to autoantigens, but also to common environmental pathogens. A similar dysregulated state, this time toward Th2 responses, is likely to exist in asthmatic or atopic individuals (24). If such a dysregulation involves abnormalities in the cAMP second messenger system, it would render TCL derived from these individuals more susceptible to the effects of PDE inhibition, thus at least in part explaining the controversies between results obtained from MS and asthmatic patients. Several reports in the literature indicate that this may be the case. Patients with MS, rheumatoid arthritis, or lupus were found to have low intracellular cAMP levels (25, 26), decreased expression and activity of G protein-coupled receptor kinases (27), or deficient type I cAMP-dependent protein kinase A activity (28, 29). Similar abnormalities in cAMP signaling were suggested in asthmatic patients (30–32).

It is more difficult to explain the suggested differential susceptibility to PDE inhibition between autoreactive and foreign Ag-reactive TCL. We did not find any significant skewing in the cytokine profiles between these two types of TCL that would account for the observed differential effect. This effect is not mediated by the need for antigenic processing of MBP compared with Flu-HA and tetanus peptides, because it was shown that MBP presentation by HLA-DR molecules does not require processing (33) and because we have noted a similar effect of PDE inhibition on seven MBP-specific TCL stimulated with the peptide epitope (data not shown). The two likely explanations are that either the signal delivered by the autoantigen may be qualitatively different (partial agonist vs full agonist signal) or the dysregulation in the cAMP system is more pronounced in autoreactive T cells. We are currently studying this issue in detail. Considering the therapeutic use of PDE inhibitors, the observed higher susceptibility of autoreactive TCL to PDE4 inhibition may widen the therapeutic window in the treatment of autoimmune disorders without inducing general immunosuppression.

To explore the possible cause of the PDE inhibitor-induced bias from Th1 to Th2 phenotype, we decided to study the influence of these drugs on T cell priming. There are three major components contributing to T cell priming, which may influence the phenotype of the primed T cell: the cytokine milieu, the dose and character of the Ag, and the costimulatory signal. It was previously demonstrated by us and others that selective PDE4 inhibitors or nonselective PDE inhibitors decrease the secretion or expression of proinflammatory cytokines by human mononuclear cells, favoring the cytokine milieu at the time of Ag presentation toward an anti-inflammatory Th2 phenotype (6, 14, 34, 35). However, the influences of PDE inhibition on the other components of T cell priming conditions were unknown. First, we studied the PDE inhibitors in Ag dose-response assays, exploring their effects on the dose of Ag required for T cell activation. The results of these studies indicate that PDE inhibitors have two different effects on the activation of TCL; they inhibit TCL proliferation regardless of the Ag dose (Fig. 4). On the other hand, the effect of PDE inhibitors on the EC_{50} indicates that a higher Ag dose is necessary for the activation of TCL under their influence. These data suggest that PDE inhibitors have complex inhibitory effects on T cell activation, most likely by influencing both proximal Ag-responsive events of T cell signaling

(36) as well as components of more downstream machinery involved in T cell effector functions. The exact mechanisms of this effect are currently under investigation in our laboratory. The demonstrated influence of PDE4 and -3 on the Ag dose required for T cell activation together with the data from the literature showing that high Ag doses skew the developing immune response toward a Th1 phenotype, whereas low doses of Ag skew the response toward a Th2 phenotype (37) adds another mechanism for the observed effect of these drugs on Th1/Th2 paradigm.

Next, we wanted to assess the influence of rolipram on the third component of T cell priming, the costimulatory signals. It has been suggested that costimulation by CD80 preferentially drives the T cell differentiation toward Th1 responses, whereas CD86 costimulation biases T cell priming toward Th2 responses (38). Although some concerns were raised regarding the general validity of this dichotomy of the roles of CD80 and CD86 (39), several reports indicate that the CD80/CD86 costimulatory system is altered in MS patients. Specifically, higher numbers of CD80$^+$ B lymphocytes in the cerebrospinal fluid (40, 41) increased serum levels of CD80$^+$ lymphocytes in patients during MS exacerbation (42), and low expression of CD86 on cerebrospinal fluid T cells (41) have been reported in patients with MS. Our data indicate that rolipram down-regulates CD80 expression and up-regulates CD86 expression on monocytes and B and T lymphocytes upon nonspecific activation with PHA or LPS. The likely explanation for this observation is the differential kinetic of induction of these costimulatory molecules on APC: CD80 is expressed later than CD86; therefore, rolipram may be preventing the switch from CD86 to CD80 expression, an issue that merits further study. We also demonstrated that rolipram down-regulates MHC class II expression, both in resting conditions and after induction by proinflammatory signals. This finding is in agreement with the observation in a murine system, where increases in intracellular cAMP inhibit the IFN-γ-mediated induction of class II MHC genes (43). Together, these changes in the costimulatory profile on APC favor T cell priming from Th1 toward a Th0 or Th2 phenotype and may limit the effective presentation of autoantigen in inflammatory MS lesions. Indeed, rolipram was shown to reduce the number of IFN-γ-secreting cells upon priming of human mononuclear cells in bulk cultures to the autoantigen MBP, while the numbers of IL-4- or IL-10-secreting cells were unaffected (44).

The above data demonstrate a favorable drug profile of PDE4 and PDE4 combined with PDE3 inhibitors for the treatment of Th1-mediated autoimmune disorders; however, the question remains of whether this immunomodulatory effect is expressed within a concentration range that is achievable in humans in vivo. The concentrations of rolipram achievable in healthy volunteers following the administration of 0.75 mg three times daily and 1.5 mg three times daily were 24 and 53 ng/ml (0.09 0.2 μM; Dr. Claus-Steffen Stuerzebecher, unpublished observations) Our data, summarized in Fig. 6, indicate that rolipram and especially the combination of rolipram and cilostamide have a mild immunomodulatory effect at the concentration of 0.1 μM. However, due to the immunomodulation at multiple levels (influence on T cell priming conditions, Ag-specific proliferation, and cytokine production) the in vivo effect is likely to be more prominent. The combination of PDE4 and PDE3 inhibitors expresses a high degree of synergism that is, to our experience, achievable even at much lower concentrations of cilostamide in the final mixture (data not shown). Therefore, the combination of PDE4 inhibitors with relatively small amounts of PDE3 inhibitors may represent a way to broaden the therapeutic window in the treatment of human disorders and should be considered for future drug development. The molecular mechanism of this synergy is not known. It has been reported that PDE3 inhibitors alone have little effect on the total intracellular cAMP levels, and they do not further enhance the cAMP accumulation induced by rolipram (45). However, it has been suggested, that PDE3 (predominantly localized to the particulate cellular fraction) and PDE4 (predominantly cytosolic) may regulate different pools of cAMP (46, 47). It is conceivable that intracellular signaling can partially adapt to the effects of PDE4 inhibition by diverting critical pathways blocked by high cytosolic cAMP concentrations and activation of PKA to the alternative pathways, which, in turn, may be affected by PDE3 inhibition. Elucidating the molecular mechanism of this synergy between PDE4 and PDE3 inhibition will enhance our understanding of cAMP second messenger signaling.

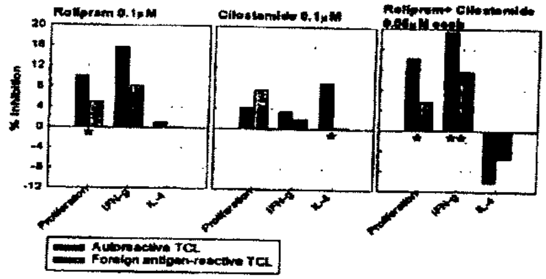

FIGURE 6. Immunomodulatory effect of PDE inhibitors within in vivo achievable concentration ranges. The concentration of rolipram that can be achieved in humans in steady state without major side effects ranges from 0.09 to 0.2 μM. At a concentration of 0.1 μM, PDE inhibitors express a mild, but multilevel, immunomodulatory profile by suppressing Ag-specific proliferation and IFN-γ production preferentially of autoreactive TCL, while not affecting, or even enhancing, IL-4 production. This immunomodulation is strongest for the combination of rolipram and cilostamide, demonstrating the synergistic effect. A statistically significant effect compared with control cultures is indicated (*, $p < 0.05$; **, $p < 0.01$).

Well-designed therapeutic trials supported by immunological studies should not only provide more definite information about the therapeutic potential of PDE inhibitors in autoimmune disorders, but also broaden our understanding of the immunopathogenesis and the potential differences among individual human Th1-mediated autoimmune diseases.

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Exhibit B

C

United States Court of Appeals,Federal Circuit.
In re Miguel F. BRANA, Jose M.C. Berlanga, Marina M. Moset, Erich Schlick and Gerhard Keilhauer.
93-1393.

March 30, 1995.

Applicants appealed from decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences, affirming patent examiner's rejections of claims for antitumor compound. The Court of Appeals, Plager, Circuit Judge, held that: (1) claimed specification for antitumor compound satisfied statutory utility requirement by alleging that compound was more effective in treating lymphocytic leukemia in mice than other known compounds; (2) PTO failed to satisfy its initial burden of challenging presumptively correct assertion of utility; (3) even if one skilled in the art would have reasonably questioned asserted utility of claimed antitumor compound, applicants provided sufficient evidence to convince one of skill in the art of asserted utility; and (4) Food and Drug Administration (FDA) approval is not prerequisite for finding compound useful within meaning of patent laws.

Reversed.

West Headnotes

[1] Patents 291 🔑101(5)

291 Patents
291IV Applications and Proceedings Thereon
291k101 Claims
291k101(5) k. Requisites and Sufficiency. Most Cited Cases
Claim specifications for antitumor compound satisfied statutory utility requirement by alleging that compound was more effective in treating lymphocytic leukemia in mice than other known compounds. 35 U.S.C.A. § 101.

[2] Patents 291 🔑48

291 Patents
291II Patentability
291II(C) Utility
291k48 k. Nature of Product or Result. Most Cited Cases
Lymphocytic leukemia tumor models used to study cancer in mice represented specific diseases against which claimed compounds in patent application could be effective, as required to satisfy statutory utility requirement, where cell lines used on models were originally derived from lymphocytic leukemias in mice and would produce that disease once implanted in mice. 35 U.S.C.A. § 101.

[3] Patents 291 🔑97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Patent and Trademark Office (PTO) has initial burden of challenging presumptively correct assertion of utility in patent disclosure. 35 U.S.C.A. § 101.

[4] Patents 291 🔑97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Only after Patent and Trademark Office (PTO) provides evidence showing that one of ordinary skill in art would reasonably doubt asserted utility of patented invention does burden shift to applicant to provide rebuttal evidence sufficient to convince such person of invention's asserted utility. 35 U.S.C.A. § 101.

[5] Patents 291 🔑97

291 Patents
291IV Applications and Proceedings Thereon
291k97 k. Patent Office and Proceedings Therein in General. Most Cited Cases
Patent and Trademark Office (PTO) failed to satisfy its initial burden of challenging presumptively correct assertion of utility in application for patent for antitumor compound, where references cited by PTO did not question usefulness of any compound as antitumor agent or provide any other evidence to cause one of skill in the art to question asserted utility of applicants' compounds, but instead discussed therapeutic predictive value of tests used in mice, which were relevant only if applicants were required

to prove ultimate value in humans of their asserted utility. 35 U.S.C.A. § 101.

[6] Patents 291 🔑99

291 Patents
291IV Applications and Proceedings Thereon
291k99 k. Description of Invention in Specification. Most Cited Cases

Even if one skilled in the art would have reasonably questioned asserted utility of claimed antitumor compound, applicants provided sufficient evidence to convince one of skill in the art of asserted utility; applicants provided test results showing that several compounds within scope of claims exhibited significant antitumor activity, and prior art disclosed structurally similar compounds which were proven to be effective antitumor agents. 35 U.S.C.A. § 101.

[7] Patents 291 🔑49

291 Patents
291II Patentability
291II(C) Utility
291k49 k. Evidence of Utility. Most Cited Cases

Although minor changes in chemical compounds can radically change their effects on human body, evidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe asserted utility.

[8] Patents 291 🔑46

291 Patents
291II Patentability
291II(C) Utility
291k46 k. Nature and Necessity of Patentable Utility. Most Cited Cases

Food and Drug Administration (FDA) approval is not prerequisite for finding compound useful within meaning of patent laws. Federal Food, Drug, and Cosmetic Act, § 505(i)(1), 21 U.S.C.A. § 355(i)(1); 35 U.S.C.A. § § 101, 112; 21 C.F.R. § § 312.21(b), 312.23(a)(5), (a)(8).

[9] Patents 291 🔑324.5

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.5 k. Scope and Extent of Review in General. Most Cited Cases

In reviewing decisions of Patent and Trademark Office (PTO), Court of Appeals traditionally reviews questions of law without deference to views of the agency, and defers to agency with regard to questions of fact unless its findings are clearly erroneous.

[10] Patents 291 🔑324.55(1)

291 Patents
291XII Infringement
291XII(C) Suits in Equity
291k324 Appeal
291k324.55 Questions of Fact, Verdicts, and Findings
291k324.55(1) k. In General. Most Cited Cases

When mixed questions of law and fact are before Court of Appeals on appeal from decision of Patent and Trademark Office (PTO), whether Court of Appeals defers, and extent to which it defers to agency's decision, turns on nature of case and nature of judgment. 5 U.S.C.A. § 706.

***1562** Malcolm J. MacDonald, Keil & Weinkauf, Washington, DC, argued, for appellant. With him on the brief was Herbert B. Keil. Of counsel was David S. Nagy.
Fred E. McKelvey, Sol., Office of Sol., Arlington, VA, argued, for appellee. With him on the brief were Albin F. Drost, Deputy Sol., Richard E. Schafer, Teddy S. Gron, Joseph G. Piccolo and Richard L. Torczon, Associate Sols.

Before PLAGER, LOURIE, and RADER, Circuit Judges.

PLAGER, Circuit Judge.
Miguel F. Brana, *et al.* (applicants), appeal the March 19, 1993 decision of the United States Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), in Appeal No. 92-1196. The Board affirmed the examiner's rejection of claims 10-13 of patent application Serial No. 533,944 under 35 U.S.C. § 112 ¶ 1 (1988).[FN1] The examiner's rejection, upon which the Board relied in rendering its decision, was based specifically on a challenge to the utility of the claimed compounds and the amount of experimentation necessary to use the compounds. We conclude the Board erred, and reverse.

FN1. Unless otherwise noted, all United States Code citations are to the 1988 edition.

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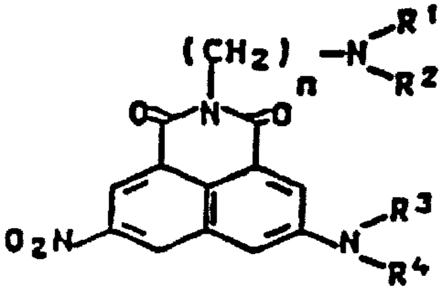
I. BACKGROUND

On June 30, 1988, applicants filed patent application Serial No. 213,690 (the '690 application) [FN2] directed to 5-nitrobenzo[de]isoquinoline-1,3-dione compounds, for use as antitumor substances, having the following formula:

> FN2. This is a divisional of patent application Serial No. 110,871 filed October 21, 1987.



where n is 1 or 2, R^1 and R^2 are identical or different and are each hydrogen, C1-C6-alkyl, C1-C6-hydroxyalkyl, pyrrolidinyl, morpholino, piperidinyl or piperacinyl, and R^3 and R^4 are identical or different and are each hydrogen, C1-C6-alkyl, C1-C6-acyl, C2-C7-alkoxycarbonyl, ureyl, aminocarbonyl or C2-C7-alkylaminocarbonyl. These claimed compounds differ from several prior art benzo[de]isoquinoline-1,3-dione compounds due to the presence of a nitro group (O2N) at the 5-position and an amino or other amino group (NR^3R^4) at the 8-position of the isoquinoline ring.

The specification states that these non-symmetrical substitutions at the 5-and 8-positions produce compounds with "a better action and a better action spectrum as antitumor substances" than known benzo[de]isoquinolines, namely those in K.D. Paull et al., *Computer Assisted Structure-Activity Correlations,* Drug Research, 34(II), 1243-46 (1984) (Paull). Paull describes a computer-assisted evaluation of benzo[de]isoquinoline-1,3-diones and related compounds which have been screened for antitumor activity by testing their efficacy ***in vivo*** [FN3] against two specific implanted murine (i.e., utilizing mice as test subjects) lymphocytic leukemias, P388 and L1210. [FN4] These two ***in vivo*** tests are *1563 widely used by the National Cancer Institute (NCI) to measure the antitumor properties of a compound. Paull noted that one compound in particular, benzo[de]isoquinoline-1,3(2H)dione,5-amino-2(2-dimethyl-aminoethyl [sic]) (hereinafter "NSC 308847"), was found to show excellent activity against these two specific tumor models. Based on their analysis, compound NSC 308847 was selected for further studies by NCI. In addition to comparing the effectiveness of the claimed compounds with structurally similar compounds in Paull, applicants' patent specification illustrates the cytotoxicity of the claimed compounds against human tumor cells, ***in vitro,*** [FN5] and concludes that these tests "had a good action." [FN6]

> FN3. ***In vivo*** means "[i]n the living body, referring to a process occurring therein." Steadman's Medical Dictionary 798 (25th ed. 1990). ***In vitro*** means "[i]n an artificial environment, referring to a process or reaction occurring therein, as in a test tube or culture media." *Id.*
>
> FN4. The analysis in Paull consisted of grouping the previously-tested compounds into groups based on common structural features and cross-referencing the various groups, in light of the success rates of the group as a whole, to determine specific compounds that may be effective in treating tumors.
>
> FN5. *See supra* note 3.
>
> FN6. The specification does not state the specific type of human tumor cells used in this test.

The examiner initially rejected applicants' claims in the '690 application as obvious under 35 U.S.C. § 103 in light of U.S. Patent No. 4,614,820, issued to and referred to hereafter as Zee-Cheng et al. Zee-Cheng et al. discloses a benzo[de]isoquinoline compound for use as an antitumor agent with symmetrical substitutions on the 5-position and 8-position of the quinoline ring; in both positions the substitution was either an amino or nitro group. [FN7] Although not identical to the applicants' claimed compounds, the examiner noted the similar substitution pattern (i.e., at the same positions on the isoquinoline ring) and concluded that a mixed substitution of the invention therefore would have been obvious in view of Zee-Cheng et al.

FN7. The chemical compound in Zee-Cheng et al. is labeled a 3,6-disubstituted-1,8-naphthalimide and uses different numbering for the positions on the isoquinoline ring. The structure of this compound, however, is identical to that claimed by the applicants except for symmetrical substitutions at the 5-position and the 8-position of the isoquinoline ring. Zee-Cheng et al. teaches identical substitutions of amino or nitro groups while applicants claim a nitro group substitution at the 5-position and an amino group substitution at the 8-position.

In a response dated July 14, 1989, the applicants rebutted the § 103 rejection. Applicants asserted that their mixed disubstituted compounds had unexpectedly better antitumor properties than the symmetrically substituted compounds in Zee-Cheng et al. In support of this assertion applicants attached the declaration of Dr. Gerhard Keilhauer. In his declaration Dr. Keilhauer reported that his tests indicated that applicants' claimed compounds were far more effective as antitumor agents than the compounds disclosed in Zee-Cheng et al. when tested, ***in vitro***, against two specific types of human tumor cells, HEp and HCT-29. [FN8] Applicants further noted that, although the differences between the compounds in Zee-Cheng et al. and applicants' claimed compounds were slight, there was no suggestion in the art that these improved results (over Zee-Cheng et al.) would have been expected. Although the applicants overcame the § 103 rejection, the examiner nevertheless issued a final rejection, on different grounds, on September 5, 1989.

FN8. HEp cells are derived from laryngeal cancer and HCT-29 cells from colon cancer.

On June 4, 1990, applicants filed a continuation application, Serial No. 533,944 (the '944 application), from the above-mentioned '690 application. Claims 10-13, the only claims remaining in the continuation application, were rejected in a final office action dated May 1, 1991. Applicants appealed the examiner's final rejection to the Board.

In his answer to the applicants' appeal brief, the examiner stated that the final rejection was based on 35 U.S.C. § 112 ¶ 1. [FN9] The examiner first noted that the specification failed to describe any specific disease against which the claimed compounds were active. Furthermore, the examiner concluded that the prior art tests performed in Paull and the tests disclosed in the specification were not sufficient to establish a reasonable expectation that the claimed compounds had ***1564** a practical utility (i.e. antitumor activity in humans). [FN10]

FN9. The examiner's answer noted that the final rejection also could have been made under 35 U.S.C. § 101 for failure to disclose a practical utility.

FN10. The examiner subsequently filed two supplemental answers in response to arguments raised by the applicants in supplemental reply briefs.

In a decision dated March 19, 1993, the Board affirmed the examiner's final rejection. The three-page opinion, which lacked any additional analysis, relied entirely on the examiner's reasoning. Although noting that it also would have been proper for the examiner to reject the claims under 35 U.S.C. § 101, the Board affirmed solely on the basis of the Examiner's § 112 ¶ 1 rejection. This appeal followed.

II. DISCUSSION

At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant prove regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago. [FN11] We note the Commissioner has recently addressed this question in his Examiner Guidelines for Biotech Applications, *see* 60 Fed.Reg. 97 (1995); 49 Pat.Trademark & Copyright J. (BNA) No. 1210, at 234 (Jan. 5, 1995).

FN11. *See, e.g., Cross v. Iizuka,* 753 F.2d 1040, 224 USPQ 739 (Fed.Cir.1985); *In re Langer,* 503 F.2d 1380, 183 USPQ 288 (CCPA 1974); *In re Krimmel,* 292 F.2d 948, 130 USPQ 215 (CCPA 1961); *In re Bergel,* 292 F.2d 958, 130 USPQ 205 (CCPA 1961).

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The requirement that an invention have utility is found in 35 U.S.C. § 101: "Whoever invents ... any new and *useful* ... composition of matter ... may obtain a patent therefor...." (emphasis added). It is also implicit in § 112 ¶ 1, which reads:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Obviously, if a claimed invention does not have utility, the specification cannot enable one to use it.

As noted, although the examiner and the Board both mentioned § 101, and the rejection appears to be based on the issue of whether the compounds had a practical utility, a § 101 issue, the rejection according to the Board stands on the requirements of § 112 ¶ 1. It is to that provision that we address ourselves. [FN12] The Board gives two reasons for the rejection; [FN13] we will consider these in turn.

1.

[1] The first basis for the Board's decision was that the applicants' specification failed to disclose a specific disease against which the claimed compounds are useful, and therefore, absent undue experimentation, one of ordinary skill in the art was precluded from using the invention. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed.Cir.1986), *cert. denied*, 480 U.S. 947, 107 S.Ct. 1606, 94 L.Ed.2d 792 (1987). In support, the Commissioner argues that the disclosed uses in ***1565** the '944 application, namely the "treatment of diseases" and "antitumor substances," are similar to the nebulous disclosure found insufficient in *In re Kirk*, 376 F.2d 936, 153 USPQ 48 (CCPA 1967). This argument is not without merit.

In *Kirk* applicants claimed a new class of steroid compounds. One of the alleged utilities disclosed in the specification was that these compounds possessed "high biological activity." *Id.* at 938, 153 USPQ at 50. The specification, however, failed to disclose which biological properties made the compounds useful. Moreover, the court found that known specific uses of similar compounds did not cure this defect since there was no disclosure in the specification that the properties of the claimed compounds were the same as those of the known similar compounds. *Id.* at 942, 153 USPQ at 53. Furthermore, it was not alleged that one of skill in the art would have known of any specific uses, and therefore, the court concluded this alleged use was too obscure to enable one of skill in the art to use the claimed invention. *See also Kawai v. Metlesics*, 480 F.2d 880, 178 USPQ 158 (CCPA 1973).

Kirk would potentially be dispositive of this case were the above-mentioned language the only assertion of utility found in the '944 application. Applicants' specification, however, also states that the claimed compounds have "a better action and a better action spectrum as antitumor substances" than known compounds, specifically those analyzed in Paull. As previously noted, *see supra* note 4, Paull grouped various benzo[de]isoquinoline-1,3-diones, which had previously been tested *in vivo* for antitumor activity against two lymphocytic leukemia tumor models (P388 and L1210), into various

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FN12. This court's predecessor has determined that absence of utility can be the basis of a rejection under both 35 U.S.C. § 101 and § 112 ¶ 1. *In re Jolles*, 628 F.2d 1322, 1326 n. 11, 206 USPQ 885, 889 n. 11 (CCPA 1980); *In re Fouche*, 439 F.2d 1237, 1243, 169 USPQ 429, 434 (CCPA 1971) ("[I]f such compositions are in fact useless, appellant's specification cannot have taught how to use them."). Since the Board affirmed the examiner's rejection based solely on § 112 ¶ 1, however, our review is limited only to whether the application complies with § 112 ¶ 1.

FN13. The Board's decision did not expressly make any independent factual determinations or legal conclusions. Rather, the Board stated that it "agree[d] with the examiner's well reasoned, well stated and fully supported by citation of relevant precedent position in every particular, and any further comment which we might add would be redundant." *Ex parte Brana et al.*, No. 92-1196 (Bd.Pat.App. & Int. March 19, 1993) at 2-3. Therefore, reference in this opinion to Board findings are actually arguments made by the examiner which have been expressly adopted by the Board.

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structural classifications and analyzed the test results of the groups (i.e. what percent of the compounds in the particular group showed success against the tumor models). Since one of the tested compounds, NSC 308847, was found to be highly effective against these two lymphocytic leukemia tumor models, [FN14] applicants' favorable comparison implicitly asserts that their claimed compounds are highly effective (i.e. useful) against lymphocytic leukemia. An alleged use against this particular type of cancer is much more specific than the vaguely intimated uses rejected by the courts in *Kirk* and *Kawai*. *See, e.g.,* Cross v. Iizuka, 753 F.2d at 1048, 224 USPQ at 745 (finding the disclosed practical utility for the claimed compounds-the inhibition of thromboxane synthetase in human or bovine platelet microsomes-sufficiently specific to satisfy the threshold requirement in *Kirk* and *Kawai.*)

FN14. Paull also found NSC 308847 to be effective against two other test models, B16 melanoma and Colon C872.

[2] The Commissioner contends, however, that P388 and L1210 are not diseases since the only way an animal can get sick from P388 is by a direct injection of the cell line. The Commissioner therefore concludes that applicants' reference to Paull in their specification does not provide a specific disease against which the claimed compounds can be used. We disagree.

As applicants point out, the P388 and L1210 cell lines, though technically labeled tumor models, were originally derived from lymphocytic leukemias in mice. Therefore, the P388 and L1210 cell lines do represent actual specific lymphocytic tumors; these models will produce this particular disease once implanted in mice. If applicants were required to wait until an animal naturally developed this specific tumor before testing the effectiveness of a compound against the tumor ***in vivo***, as would be implied from the Commissioner's argument, there would be no effective way to test compounds ***in vivo*** on a large scale.

We conclude that these tumor models represent a specific disease against which the claimed compounds are alleged to be effective. Accordingly, in light of the explicit reference to Paull, applicants' specification alleges a sufficiently specific use.

2.

[3] [4] The second basis for the Board's rejection was that, even if the specification did allege a specific use, applicants failed to ***1566** prove that the claimed compounds are useful. Citing various references, [FN15] the Board found, and the Commissioner now argues, that the tests offered by the applicants to prove utility were inadequate to convince one of ordinary skill in the art that the claimed compounds are useful as antitumor agents. [FN16]

FN15. *See* Pazdur et al., *Correlation of Murine Antitumor Models in Predicting Clinical Drug Activity in Non-Small Cell Lung Cancer: A Six Year Experience,* 3 Proceedings Am.Soc.Clin.Oncology 219 (1984); Martin et al., *Role of Murine Tumor Models in Cancer Research,* 46 Cancer Research 2189 (April 1986).

FN16. As noted, this would appear to be a § 101 issue, rather than § 112.

This court's predecessor has stated:

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). From this it follows that the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. *Id.* at 224, 169 USPQ at 370. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy,* 642 F.2d 430, 433, 209 USPQ 48, 51 (CCPA 1981). [FN17]

FN17. *See also In re Novak,* 306 F.2d 924, 928, 134 USPQ 335, 337 (CCPA 1962) (stating that it is proper for the examiner to request evidence to substantiate an asserted

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> utility unless one with ordinary skill in the art would accept the allegations as obviously valid and correct); *In re Chilowsky,* 229 F.2d 457, 462, 108 USPQ 321, 325 (CCPA 1956) ("[W]here the mode of operation alleged can be readily understood and conforms to the known laws of physics and chemistry ... no further evidence is required."). *But see In re Marzocchi,* 439 F.2d at 223, 169 USPQ at 369-70 ("In the field of chemistry generally there may be times when the well-known unpredictability of chemical reactions will alone be enough to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim. This will especially be the case where the statement is, on its face, contrary to generally accepted scientific principles.").

[5] The PTO has not met this initial burden. The references cited by the Board, Pazdur and Martin, [FN18] do not question the usefulness of any compound as an antitumor agent or provide any other evidence to cause one of skill in the art to question the asserted utility of applicants' compounds. Rather, these references merely discuss the therapeutic predictive value of *in vivo* murine tests-relevant only if applicants must prove the ultimate value in humans of their asserted utility. Likewise, we do not find that the nature of applicants' invention alone would cause one of skill in the art to reasonably doubt the asserted usefulness.

The purpose of treating cancer with chemical compounds does not suggest an inherently unbelievable undertaking or involve implausible scientific principles. *In re Jolles,* 628 F.2d at 1327, 206 USPQ at 890. Modern science has previously identified numerous successful chemotherapeutic agents. In addition, the prior art, specifically Zee Cheng et al., discloses structurally similar compounds to those claimed by the applicants which have been proven *in vivo* to be effective as chemotherapeutic agents against various tumor models.

Taking these facts-the nature of the invention and the PTO's proffered evidence-into consideration we conclude that one skilled in the art would be without basis to reasonably doubt applicants' asserted utility on its face. The PTO thus has not satisfied its initial burden. Accordingly, applicants should not have been required to substantiate their presumptively correct disclosure to avoid a rejection under the first paragraph of § 112. *See In re Marzocchi,* 439 F.2d at 224, 169 USPQ at 370.

[6] We do not rest our decision there, however. Even if one skilled in the art *1567 would have reasonably questioned the asserted utility, i.e., even if the PTO met its initial burden thereby shifting the burden to the applicants to offer rebuttal evidence, applicants proffered sufficient evidence to convince one of skill in the art of the asserted utility. In particular, applicants provided through Dr. Kluge's declaration [FN19] test results showing that several compounds within the scope of the claims exhibited significant antitumor activity against the L1210 standard tumor model ***in vivo.*** Such evidence alone should have been sufficient to satisfy applicants' burden.

[7] The prior art further supports the conclusion that one skilled in the art would be convinced of the applicants' asserted utility. As previously mentioned, prior art-Zee Cheng et al. and Paull-disclosed structurally similar compounds which were proven ***in vivo*** against various tumor models to be effective as chemotherapeutic agents. Although it is true that minor changes in chemical compounds can radically alter their effects on the human body, *Kawai,* 480 F.2d at 891, 178 USPQ at 167, evidence of success in structurally similar compounds is

FN18. *See supra* note 15.

FN19. The declaration of Michael Kluge was signed and dated June 19, 1991. This declaration listed test results (i.e. antitumor activity) of the claimed compounds, *in vivo,* against L1210 tumor cells and concluded that these compounds would likely be clinically useful as anti-cancer agents. Enablement, or utility, is determined as of the application filing date. *In re Glass,* 492 F.2d 1228, 1232, 181 USPQ 31, 34 (CCPA 1974). The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. *In re Marzocchi,* 439 F.2d at 224 n. 4, 169 USPQ at 370 n. 4. It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility).

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relevant in determining whether one skilled in the art would believe an asserted utility. *See Rey-Bellet v. Engelhardt*, 493 F.2d 1380, 181 USPQ 453 (CCPA 1974); *Kawai*, 480 F.2d 880, 178 USPQ 158.

The Commissioner counters that such *in vivo* tests in animals are only preclinical tests to determine whether a compound is suitable for processing in the second stage of testing, by which he apparently means *in vivo* testing in humans, and therefore are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. FN20 The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption. *See Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed.Cir.1994) ("Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.").

> FN20. We note that this discussion is relevant to the earlier discussion as well. If we were to conclude that these *in vivo* tests are insufficient to establish usefulness for the claimed compounds, that would bear on the issue of whether one skilled in the art would, in light of the structurally similar compounds in Paull and Zee Cheng et al., have cause to doubt applicants' asserted usefulness for the compounds.

Our court's predecessor has determined that proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel*, 292 F.2d 948, 953, 130 USPQ 215, 219 (CCPA 1961); *see also In re Bergel*, 292 F.2d 958, 130 USPQ 205 (CCPA 1961). In concluding that similar *in vivo* tests were adequate proof of utility the court in *In re Krimmel* stated:

We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment in humans.

Krimmel, 292 F.2d at 953, 130 USPQ at 219. Moreover, NCI apparently believes these tests are statistically significant because it has explicitly recognized both the P388 and L1210 murine tumor models as standard screening tests for determining whether new ***1568** compounds may be useful as antitumor agents.

In the context of this case the Martin and Pazdur references, on which the Commissioner relies, do not convince us otherwise. Pazdur only questions the reliability of the screening tests against lung cancer; it says nothing regarding other types of tumors. Although the Martin reference does note that some laboratory oncologists are skeptical about the predictive value of ***in vivo*** murine tumor models for human therapy, Martin recognizes that these tumor models continue to contribute to an increasing human cure rate. In fact, the authors conclude that this perception (i.e. lack of predictive reliability) is not tenable in light of present information.

On the basis of animal studies, and controlled testing in a limited number of humans (referred to as Phase I testing), the Food and Drug Administration may authorize Phase II clinical studies. *See* 21 U.S.C. § 355(i)(1); 21 C.F.R. § 312.23(a)(5), (a)(8) (1994). Authorization for a Phase II study means that the drug may be administered to a larger number of humans, but still under strictly supervised conditions. The purpose of the Phase II study is to determine primarily the safety of the drug when administered to a larger human population, as well as its potential efficacy under different dosage regimes. *See* 21 C.F.R. § 312.21(b).

[8] FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. *Scott*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

In view of all the foregoing, we conclude that applicants' disclosure complies with the requirements of 35 U.S.C. § 112 ¶ 1.

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3.

[9] The Commissioner takes this opportunity to raise the question of this court's standard of review when deciding cases on appeal from the PTO. Traditionally we have recited our standard of review to be, with regard to questions of law, that review is without deference to the views of the Agency, *In re Donaldson*, 16 F.3d 1189, 1192, 29 USPQ2d 1845, 1848 (Fed.Cir.1994) (in banc), *In re Caveney*, 761 F.2d 671, 674, 226 USPQ 1, 3 (Fed.Cir.1985), and with regard to questions of fact, we defer to the Agency unless its findings are "clearly erroneous." *See, e.g., In re Baxter Travenol Labs*, 952 F.2d 388, 21 USPQ2d 1281 (Fed.Cir.1991); *In re Woodruff*, 919 F.2d 1575, 16 USPQ2d 1934 (Fed.Cir.1990); *In re De Blauwe*, 736 F.2d 699, 222 USPQ 191 (Fed.Cir.1984).

[10] With regard to judgment calls, those questions that fall "[s]omewhere near the middle of the fact-law spectrum," this court has recognized "the falseness of the fact-law dichotomy, since the determination at issue, involving as it does the application of a general legal standard to particular facts, is probably most realistically described as neither of fact nor law, but mixed." *Campbell v. Merit Systems Protection Board*, 27 F.3d 1560, 1565 (Fed.Cir.1994). When these questions of judgment are before us, whether we defer, and the extent to which we defer, turns on the nature of the case and the nature of the judgment. *Id.* ("Characterization therefore must follow from an *a priori* decision as to whether deferring ... is sound judicial policy. We would be less than candid to suggest otherwise.").

The Commissioner contends that the appropriate standard of review for this court regarding questions of law, of fact, and mixed questions of law and fact, coming to us from the PTO is found in the Administrative Procedure Act (APA) at 5 U.S.C. § 706. The standard set out there is that "[t]he reviewing court shall ... hold unlawful and set aside agency action, findings, and conclusions found to be-(A) arbitrary, capricious, an ***1569** abuse of discretion, or otherwise not in accordance with law; ... (E) unsupported by substantial evidence...." The Commissioner is of the view that the stated standard we now use, which is the traditional standard of review for matters coming from a trial court, is not appropriate for decisions coming from an agency with presumed expertise in the subject area, and is not in accord with law. [FN21]

Applicants argue that by custom and tradition, recognized by the law of this court, the standard of review we have applied, even though inconsistent with the standard set forth in the APA, nevertheless is a permissible standard. In our consideration of this issue, there is a reality check: would it matter to the outcome in a given case which formulation of the standard a court articulates in arriving at its decision? The answer no doubt must be that, even though in some cases it might not matter, in others it would, otherwise the lengthy debates about the meaning of these formulations and the circumstances in which they apply would be unnecessary.

A preliminary question, then, is whether this is one of those cases in which a difference in the standard of review would make a difference in the outcome. The ultimate issue is whether the Board correctly applied the § 112 ¶ 1 enablement mandate and its implicit requirement of practical utility, or perhaps more accurately the underlying requirement of § 101, to the facts of this case. As we have explained, the issue breaks down into two subsidiary issues: (1) whether a person of ordinary skill in the art would conclude that the applicants had sufficiently described particular diseases addressed by the invention, and (2) whether the Patent Act supports a requirement that makes human testing a prerequisite to patentability under the circumstances of this case.

The first subsidiary issue, whether the application adequately described particular diseases, calls for a judgment about what the various representations and discussions contained in the patent application's specification would say to a person of ordinary skill in the art. We have considered that question carefully, and, for the reasons we explained above in some detail, we conclude that the Board's judgment on this question was erroneous. Our conclusion rests on our understanding of what a person skilled in

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FN21. Congress enacted the Administrative Procedure Act (APA) on June 11, 1946. *See* 1 Kenneth Culp Davis, Administrative Law Treatise, § 1:7 (2d ed. 1978). The APA sets forth a framework for administrative agency procedure and provides judicial review for persons adversely affected by final agency actions. Chapter 7, codified at 5 U.S.C. § 701-706, contains the APA judicial review provisions, including the standard of review provision quoted above.

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the art would gather from the various art cited, and from the statements in the application itself. We consider the Board's error to be sufficiently clear that it is reversible whether viewed as clear error or as resulting in an arbitrary and capricious decision.

The second subsidiary issue, whether human testing is a prerequisite to patentability, is a pure question of law: what does the practical utility requirement mean in a case of this kind. Under either our traditional standard or under the APA standard no deference is owed the Agency on a question of law, and none was accorded.

If the question concerning the standard of review, raised by the Commissioner, is to be addressed meaningfully, it must arise in a case in which the decision will turn on that question, and, recognizing this, the parties fully brief the issue. This is not that case. We conclude that it is not necessary to the disposition of this case to address the question raised by the Commissioner; accordingly, we decline the invitation to do so.

III. CONCLUSION

The Board erred in affirming the examiner's rejection under 35 U.S.C. § 112 ¶ 1. The decision is reversed.

REVERSED.

C.A.Fed.,1995.
In re Brana
51 F.3d 1560, 34 U.S.P.Q.2d 1436, 63 USLW 2656

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Exhibit C

Current Pharmaceutical Design, 2002, 8, 1255-1296

Recent Advances in PDE4 Inhibitors as Immunoregulators and Anti-Inflammatory Drugs

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Abstract: The phosphodiesterases (PDEs) are responsible for the hydrolysis of intracellular cyclic adenosine and guanosine monophosphate (cAMP and cGMP, respectively). They are classified into 11 major families (PDE1-11) and the type 4 phosphodiesterase (PDE4) is a cAMP-specific enzyme localized in airway smooth muscle cells as well as in immune and inflammatory cells. The PDE4 activity is associated with a wide variety of diseases some of which have been related to an inflammatory state, (e.g. asthma, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis (RA)) while others have recently been connected to autoimmune pathology. Therefore, an intense effort toward the development of PDE4 inhibitors has been generated for the last decade. Unfortunately, the effects of prototype PDE4 inhibitors have been compromised by side effects such as nausea and emesis and the clinical use of those compounds is still limited. Several companies have focused on the design of a new generation of PDE4 inhibitors dissociating beneficial activity and adverse effects. This review highlights the recent data of the most advanced clinical candidates, the design and structure activity relationships of the recent structural series reported in the literature over the last two years, as well as recent advances in the multiple therapeutic indications of PDE4 inhibitors (a review with 375 references).

INTRODUCTION

The cyclic nucleotides cAMP and cGMP are intracellular second messengers that mediate many physiological processes. At high concentration these nucleotides bind to specific protein kinases (PKA and PKB). The subsequent activation of these kinases induces the phosphorylation of a wide variety of substrates that modify cellular responses. The levels of the cyclic nucleotides are regulated by the rates of the related synthesis and degradation: the synthesis is generated from ATP and GTP by adenylate and guanylate cyclases that are activated by G-protein coupled receptors and the degradation is linked to phosphodiesterase activity. The PDEs comprise a large superfamily of proteins whose members are responsible for the hydrolysis of cAMP and cGMP to the corresponding inactive nucleoside 5'-monophosphates AMP and GMP, respectively. The classification of PDE enzymes into 11 families with several different species including subtypes and splice variants has been described [1-3]. Very recently, much progress in the PDE field has been provided with the discovery and the characterization of several new phosphodiesterase genes [4] including PDE7 [5-7], PDE8 [8-9], PDE9 [10], PDE10 [11-15] and PDE11 [16-17]. The availability of phosphodiesterase isoenzymes as pure recombinant proteins has greatly facilitated the identification of potent, selective inhibitors. The potential of these inhibitors to therapeutically exploit the molecular diversity of the phosphodiesterases has progressed significantly [18].

cAMP specific PDE4 has emerged as the predominant isoenzyme in inflammatory cells and in pulmonary smooth muscle cells. The PDE4 family is comprised of four genes (PDE4A to D) [19] with a unique chromosomal location per gene [20-21]. Due to alternative splicing of the genes, multiple splice variants are reported and classified into two main groups, the long and short forms. All subtypes provide a conserved catalytic domain (~270 amino acids) and the absence or presence, respectively for short and long form, of two additional regions UCR1 (upstream conserved region 1) and UCR2 within the N-terminus [22-23]. The four subtypes are found differentially expressed among tissues and cells [24-25]. PDE4A, PDE4B and PDE4D gene products are found in most immune and inflammatory cells [25-32]. PDE4A, PDE4D and PDE4C gene are expressed in airway epithelial cells [33-34]. The four genes are expressed in the brain showing a differential distribution within the regions [31, 35-36].

During the last decade, numerous preclinical studies have demonstrated the modulation of inflammatory and immunocompetent cell activation by selective PDE4 inhibitors, supporting their potential use as emerging therapies in various inflammatory and immune diseases. More recently, a few clinical trials have confirmed the hypothesis that PDE4 inhibitors may provide benefit in the treatment of chronic airway diseases such as asthma and COPD. This review highlights the most recent advances in the design of PDE4 inhibitors as a new class of anti-inflammatory and immunomodulatory drugs.

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EFFECTS ON INFLAMMATORY CELLS

The recruitment of activated inflammatory cells at the site of injury is one of the key characteristics of inflammation. While this process remains essential for the homeostasis, in some particular conditions, cell recruitment becomes excessive and contributes to the worsening and the chronicity of the disease. Activation of inflammatory cells is characterized by the release of various pro-inflammatory mediators such as histamine, leukotrienes, granule proteins, oxygen-derived metabolites, cytokines which exert a deleterious effect on effector tissues. PDE4 is the most represented PDE in inflammatory cells and the inhibition of PDE4 activity has been extensively studied in these cell types generating a wide range of *in vitro* and *in vivo* data.

Basophils

PDE3, PDE4 and PDE5 are present in human basophils [37]. Selective inhibition of PDE4 in this cell type leads to the down-regulation of anti-immunoglobulin E (IgE)-induced histamine and leukotriene C_4 (LTC_4) release [38-39] with a maximum of activity around 50 to 60% inhibition. An inhibitory effect on interleukin (IL)-13 and IL-4 release from mite allergen-stimulated asthmatic human basophils was also described with rolipram [40].

Mast Cells

Few data only are available regarding the effects of non selective PDE inhibitors in primary human mast cells, where PDE3, PDE4 and PDE5 are all expressed. For example, 3-isobutyl-1-methylxanthine (IBMX) produces a strong inhibition of anti IgE-induced cysteinyl leukotriene and prostaglandin D_2 (PGD_2) release from mast cells, an effect that is reported not to be due to PDE4 inhibition [39]. In mast cell like cultured human cord blood mononuclear cells, selective PDE4 inhibitors inhibit granulocyte macrophage-colony stimulating factor (GM-CSF), IL-5 and macrophage inflammatory protein 1α (MIP1α) release but not histamine or PGD_2 release following anti-IgE stimulation [41-42]. No effects are reported regarding proteases, platelet-activating factor (PAF), adenosine, tumor necrosis factor α (TNFα) and other mast cell derived cytokines.

Eosinophils

Both PDE3 and PDE4 are represented in human eosinophils. Particularly, PDE4A, B and D subtypes but not PDE4C are expressed in this cell type [29]. Eosinophils, that play an important role in the pathophysiology of asthma, have been extensively studied in the context of PDE4 inhibition. Selective PDE4 inhibitors block superoxide and reactive oxygen species formation [43-47] and LTC_4 release [48-49]. The production of granule proteins such as eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) is down-regulated by PDE4 inhibitors alone [50-51] while the inhibition of eosinophil peroxidase (EPO) release requires a combination of the PDE4 inhibitor with salbutamol [52]. Complement factor C5a-, PAF- and eotaxin-induced eosinophil chemotaxis as well as PAF-, eotaxin- and GM-CSF-induced CD11b (α_M subunit of Mac 1) up-regulation can also be inhibited by PDE4 inhibitors [51, 53-54]. Finally, the effect of PDE4 inhibitors on IL-5-induced eosinophil survival was also demonstrated [51]. *In vivo*, the effect of PDE4 inhibitors on eosinophil recruitment was even more studied using different models. In sensitized and ovalbumin challenge mice, PDE4 inhibitors significantly block eosinophil but also lymphocyte and neutrophil recruitments, prevent IL-4 and IL-5 accumulation in the bronchoalveolar lavage (BAL) fluids and changes in airway functions [55]. PDE4 inhibitors also suppress sephadex- [56], respiratory syncitial virus (RSV)-induced [57], LTD_4- [58], IL-5 [59] and antigen-induced eosinophil infiltration into the lungs of guinea pigs [45, 58, 60-62], rats [63] ferrets [64] and monkeys [65-66].

Neutrophils

Neutrophils play an important role in inflammatory diseases, accumulating rapidly at the site of injury or infection. In neutrophils, cAMP is primarily hydrolyzed by PDE4 and PDE4B2 is the predominent variant [67]. PDE4 inhibitors suppress a range of neutrophil functions, including superoxide production [38, 68], granule secretion [38], IL-8 release [69], adhesion molecule expression [54], chemotaxis and adhesion [68, 70], human lung elastase release and expression [71] and LTB_4 biosynthesis [49, 72] but do not compromise phagocytosis or microbial killing [68]. Inhibition of PDE4 with appropriate activation of adenylate cyclase is sufficient to inhibit induction of E-selectin expression on human lung microvascular endothelial cells to a level that has functional consequences for neutrophil adhesion [73]. *In vivo*, PDE4 inhibitors block lipopolysaccharide (LPS)-induced neutrophilia in bronchoalveolar lavages of rats [74-75] and guinea pigs [76-77], ovalbumin-induced BAL neutrophilia in sensitized mice [55] and rats [78] and LPS-induced [^{111}In]-polymorphonuclear cell trapping in the lungs of rabbits [79].

Monocytes/Macrophages

The importance of mononuclear phagocytes in inflammation is due to their complementary and interrelated functions, blood monocytes being the circulating precursors of tissue macrophages. While PDE4 is predominent in human monocytes, PDE3 activity increases and PDE1 and 5 appear when monocytes differenciate into macrophages [80-82]. One of the major effects of PDE4 inhibitors is probably their ability to down-regulate LPS-induced TNFα production. While this inhibitory effect on TNFα is complete and extremely potent in monocytes [38, 83] it becomes less pronounced in macrophages [47, 80] probably due the decline of PDE4 activity in this cell type. One study reports an increase in total cAMP PDE activity in monocytes taken from mild asymptomatic asthmatics compared to healthy subjects, reflected by an increase in the proportion of PDE3 and a decrease in the proportion of PDE4. However, this augmented enzyme activity was not associated with an alteration in the ability of PDE4 inhibitors to prevent TNFα release from monocytes in asthmatics compared to healthy individuals [84]. PDE4 activity is upregulated in activated human peripheral blood mononuclear cells (PBMCs) [85]; furthermore, in human monocytes activated by LPS, it was suggested that only the PDE4B gene was induced and that this upregulation could be inhibited by IL-10 [67, 86]. PDE4 inhibitors block in a lesser extent the release of IL-1β [87-88] or GM-CSF [89] from primary monocytes or human PBMCs stimulated with LPS. Contradictory data are reported on IL-10 release with either an enhancing effect of PDE4 inhibitors when PBMCs are stimulated with LPS [90] or an inhibitory effect when PBMCs are stimulated with phytohemagglutinine (PHA) [91]. The production of LTB_4, LTC_4 and the release of arachidonate from human monocytes are also affected by PDE4 inhibitors [92-93]. A few studies on human macrophages report little or no effects of selective PDE4 inhibitors on respiratory burst, thromboxane B_2 (TXB_2) or LTB_4 release [94-96] while in guinea pigs alveolar macrophages, significant inhibitory effects were shown on arachidonate release [97]. *In vivo*, numerous studies report the ability of PDE4 inhibitors to reduce TNFα release in the blood or in BAL fluids of various species [63, 75-76, 98].

Endothelial Cells

Endothelial cells play a major role during inflammation by allowing inflammatory and immunocompetent cell to migrate from the circulation to the inflamed tissues. This process includes the increased permeability of endothelial layers and the up-regulation of adhesion molecules on the surface of endothelial cells. Inhibition of PDE4 with appropriate activation of adenylate cyclase is sufficient to inhibit induction of E-selectin expression on human lung microvascular endothelial cells (HLMVEC) to a level that has functional consequences for neutrophil adhesion. In contrast, combined inhibition of PDE3 and 4 isoenzymes is necessary to block vascular cell adhesion molecule (VCAM)-1 and to have inhibitory effects on eosinophil adhesion to activated HLMVEC. Finally, upregulation of intercellular adhesion molecule (ICAM)-1 expression on HLMVEC does not appear to be modulated by PDE3 and PDE4 inhibition [73]. Similarly in human vascular endothelial cells in culture, rolipram in combination with forskolin inhibits TNFα-, LPS- or phorbol myristate aceter (PMA)-induced E-selectin expression but has little effect on ICAM-1 expression [99]. The effects of PDE4 inhibitors on plasma extravasation were mainly studied in *in vivo* models of pulmonary inflammation in rats, mice and guinea-pigs. PDE4 inhibitors suppress leakage induced by several stimuli, including antigen, PAF, histamine, LPS and IL-2 [100-105].

Epithelial Cells

Epithelial cells actively participate in inflammatory disease by liberating mediators such as arachidonate metabolite and cytokines. Human bronchial epithelial cells express a low total PDE activity, made up predominantly of PDE1 and PDE4 [106]. PDE5 as well as PDE7 are also detected in primary cultures of human airway epithelium. In these cells, enhanced cAMP levels, induced by forskolin and PDE4 inhibition, increased the formation of PGE_2, but not of IL-8 or 15-hydroxyeicosatetraenoic acid (15-HETE) whereas increased cGMP levels provoked by PDE5 inhibition reduced the PGE_2 synthesis [34]. A selective PDE4 inhibitor can significantly block TNFα and GM-CSF but not IL-8 release from bronchial epithelial cells isolated from COPD patients [107]. *In vivo*, PDE4 inhibitors have been reported to exert a protective action on epithelium and to modulate tissue remodeling following ozone or antigen exposure, probably by down-regulating the activity of various proteins such as matrix metalloproteinase (MMP)-9 and IL-6 [76, 108-109].

EFFECTS ON IMMUNE CELLS

Dendritic Cells

Only few studies report the effects of selective PDE4 inhibitors on dendritic cells. During differenciation of human monocytes to dendritic cells, a drastic decrease in PDE4 activity was observed, while activities of PDE1 and PDE3 substantially increased. In these cells, selective PDE4 inhibitors block the production of TNFα in response to LPS stimulation. Moreover, rolipram, in combination with a selective PDE3 inhibitor produced a synergistic inhibitory effect on CD4+ T cell proliferation in response to antigen presentation by dendritic cells [47, 110].

Lymphocytes

T lymphocytes play a key role in coordinating the immune response. These cells express PDE4, PDE1, PDE3, PDE5 and PDE7. The distribution of PDE4 activity appears to be similar between CD4+ and CD8+ lymphocytes [29] but elevated levels of PDE4 mRNA as well as increased cAMP PDE activity have been reported in the CD4+ T cells of atopic donors compared to non atopic [111-112]. Another study reports the presence of PDE4A, PDE4B and PDE4D isoforms in CD4+ and CD8+ lymphocytes with no significant differences observed between healthy and asthmatic subjects [113]. While the expression of the various PDE4 isoforms has been identified in T helper (Th)-1 and Th-2 clones [114], it is suggested that Th-2 antigen-specific T-cell clones are more sensitive to PDE4 inhibition than Th-1 antigen-specific clones [115] and that PDE4A and PDE4B play a major role against T cell proliferation [83]. In general, PDE4 inhibitors prevent anti CD3/anti CD28-, PHA-, PMA- or antigen-induced T cell proliferation, IL-2, IL-4, IL-5, IL-13 interferon (INF)-γ production [38, 47, 116-121], PAF and IL-8 induced T cell chemotaxis [122]. IL-10 production from human PBMCs was shown to be reduced by PDE4 inhibitors, probably indirectly as a result of their inhibitory effect on TNFα release [91].

The effects of PDE4 inhibitors on B lymphocytes have been less studied. B cells contain majoritary PDE4 and PDE3 [29, 123]. Recent studies report inhibitory effects of rolipram on IL-4-induced IgE synthesis from human PBMCs, but no effects when rolipram is incubated with purified B cells only [124] suggesting a mediation via monocytes or T cells. Interestingly, B cell proliferation following stimulation with LPS and IL-4 was potentiated by rolipram [125]. Finally, no effects have been reported so far on other immunoglobulins.

THERAPEUTIC POTENTIAL IN INFLAMMATORY AND IMMUNE DISEASES

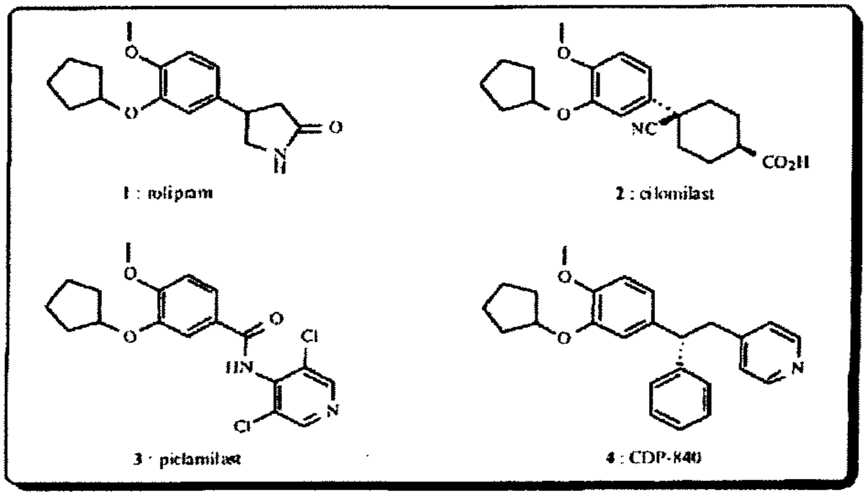
Because of the dual capacity of cAMP to down-regulate inflammatory and immune cell activation and to mediate smooth muscle cell relaxation, PDE4 inhibitors have first been designed for the treatment of chronic inflammation of the airways such as asthma and COPD [126-130]. While PDE4 inhibitors are relatively poor bronchodilators compared to β_2-adrenoceptor agonists, their potential as agents susceptible to treat the inflammation underlying chronic airway diseases has gained the interest of numerous pharmaceutical companies and has been the subject of several recent reviews [131-139]. More recently, their potential as immunomodulatory agents has gained further interest [140-141] and several hypotheses have been built regarding their role in allergic disorders [142], autoimmune diseases [143] and other inflammatory diseases [144-158].

The therapeutic potential of PDE4 inhibitors has been extensively investigated in preclinical models of rheumatoid arthritis (RA) [159], atopic dermatitis [160], multiple sclerosis [161-165], Crohn's disease [166], colitis [167], acute lung injury [168], inflammatory pain [169-170], lymphoid malignancies [171-173], hypertension [174], osteopenia [175], erectile dysfunction [176], anxiety [177], diabetes [178] and even infections with HIV [179-180].

DRUG DESIGN STRATEGIES

Clinically, the effects of PDE4 inhibitors including the prototypical rolipram (1) have been limited by side effects such as nausea and vomiting that occurred at therapeutic plasma levels [181-183]. Other effects have been reported, mainly such as increased gastric secretion and CNS-driven side effects [182, 184-188]. To date, no selective PDE4 inhibitor is on the market. Rolipram, early discovered as a potent and selective PDE4 inhibitor, was the main starting template used for the synthesis of new derivatives [189]. A wide variety of analogs were synthesized but unfortunately providing more or less similar side effect profile than their precursor rolipram. In the last decade, companies with an interest in the PDE4 area have focused on the design of a new generation of candidates providing an improved therapeutic window dissociating beneficial anti-inflammatory activity and emetic side effects. For this purpose, various strategies still are under investigation.

One of the first strategies is the identification of inhibitors structurally unrelated to rolipram [190]. Another strategy focuses on the discovery of candidates selectively inhibiting one of the two conformers of PDE4. This approach is based on the discovery that PDE4 may exist in two different conformations. Historically, it was reported that in addition to binding at the catalytic site of PDE4 (Ki ~1 µM), rolipram was found to bind an additional site with high affinity (Ki ~1 nM) named HARBS (High Affinity Rolipram Binding Site) [189]. Further scientific debate concluded that the two binding sites represent two conformers of PDE4 that were termed LPDE4 (Low affinity rolipram binding PDE4) or LAR (Low Affinity for Rolipram) and HPDE4 (High affinity rolipram binding PDE4) or HAR (High Affinity for Rolipram) [131-132, 137, 191-194]. Using a novel fluorescence resonance energy transfer (FRET)-based equilibrium PDE4 binding assay, recent data demonstrated that the two conformations result from the reversible PDE4 binding to a cofactor (Mg^{2+}). In this study, cAMP and PDE4 inhibitors including (R)-rolipram, cilomilast (2), piclamilast (3) and CDP-840 (4) displayed a differential binding affinity to the apoenzyme (free enzyme) and to the holoenzyme (enzyme bound to Mg^{2+}). The Mg^{2+} binding induces a PDE4 conformational change that produces a high affinity interaction with cAMP and therefore activates the catalytic mechanism [195]. It was suggested that HPDE4 conformation predominates in CNS



1 : rolipram

2 : cilomilast

3 : piclamilast

4 : CDP-840

[196], whereas LDPE4 is much more important than HPDE4 in immune and inflammatory cells [197-199]. Several studies (but not all) suggested that PDE4 inhibitors associated to LPDE4 conformation are related to beneficial activity [131, 191, 200-205] while candidates being connected to the HPDE4 conformation provide various PDE4 adverse effects [194, 206-210].

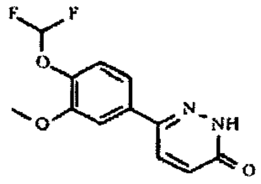
The presence of multiple subtypes and splice variants, coupled with differential expression and regulation among tissues and cells, provides a wide range of opportunities for the development of a new generation of selective inhibitors that should dissociate between beneficial activity and adverse effects. Therefore, a related strategy attempts to identify inhibitors targeting one PDE4 subtype that could lead to increase tissue and cell selectivity, to decrease side effects and finally to improve the therapeutic index [23-24, 211]. However, there are challenges associated with this approach: firstly, the high sequence homology between the subtypes and secondly the choice of the subtype to be targeting [23]. Cilomilast (2) is a unique compound in clinical phase trials providing a weak PDE4D selectivity, 10 fold versus other PDE4 subtypes [212].

A different route for reducing PDE4 side effects concerns an inhaled administration of inhibitors in order to decrease systemic exposure [213]. However, the mixed PDE3/PDE4 inhibitor zardaverine (5) is associated with some PDE4 specific events of nausea and vomiting [214]. Zardaverine clinical development was discontinued and the approach using inhaled PDE4 inhibitors is not without its challenges.

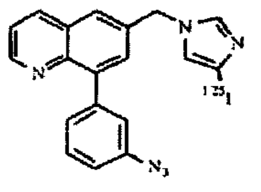
Very recently, data showed that the disease activated drug (DAD) concept could lead to a novel system for treating chronic inflammatory diseases with a reduced side effect profile. DAD drugs are a derivatization of one active principle or a combination of two covalently linked drugs. DAD drugs in contrast to prodrugs release their active principle(s) only when they reach the inflamed tissues. Thus, this approach is supposed to show many advantages including the diminution of side effects. The DAD concept was evaluated in asthma using an isoquinoline derivative as an inhaled PDE4 inhibitor. This inhibitor was either derivatized into prodrugs or combined with corticosteroids [215]. In fact, reduced systemic side effects of inhaled DADs were observed and might be associated to the local release of the active moiety in inflamed lung. Moreover, using DAD as a combination between a PDE4 inhibitor and a steroid in clinic, a reduced clinical effective dose was observed and reported as a result of a synergy of potencies connected to the liberation of the two anti-inflammatory actors.

Studies from Merck Frost suggested that, at least in ferret, PDE4 inhibitors through the cAMP elevation in sympathetic neurons induce a similar emetic response to alpha$_2$-adrenoceptor antagonists. The authors suggested that PDE4 inhibitors increasing intracellular levels of cAMP may modulate the level of mediators such as noradrenaline, 5-HT and substance P and may be responsible for inducing side effects [216-218]. Therefore, emesis produced by PDE4 inhibitors may be evaluated through the appropriate emetic response in ferrets or through the anesthetic reversal effect in rats. Merck Frost was the first group having identified a photoaffinity probe (6) emetic in ferrets, efficacious and competitive with other PDE4 inhibitors [219]. This probe should be useful for the identification of the emesis and efficacy targets of PDE4 inhibitors.

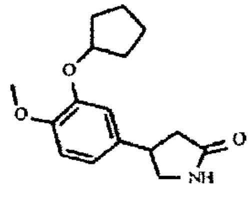
Inflazyme described a method for enhancing the therapeutic activity of PDE4 inhibitors by reducing their emetogenic properties [220]. The method is made up of a benzylation of PDE4 inhibitors: suitable benzylation chemistry is to extract a hydrogen from a PDE4 inhibitor, preferably with a base, and then react the resulting nucleophilic PDE4 inhibitor with a benzylating agent. The benzyl group may be attached to either a carbon or nitrogen



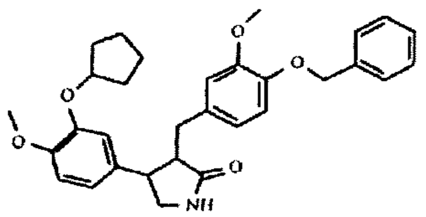
5 : zardaverine



6 : PDE4 radiolabeled inhibitor



1 : rolipram



7

atom of a PDE4 inhibitor. The benzylated rolipram analog (7) was > 10-fold less emetic than rolipram. It was potent as rolipram on PDE4 enzyme with IC_{50} values of 0.40 and 0.42 μM but provided a 60-fold lower affinity for the HARBS with respective IC_{50}s of 0.36 and 0.0065 μM. The synthesis of several reference PDE4 inhibitors was reported.

The 3D-structure of the catalytic domain of PDE4B2B was recently resolved [221]. The catalytic domain folds into a novel compact structure composed of 17 helices and 3 subdomains. The PDE4B2B crystal structure, as a representative of this therapeutically important class of enzymes, provides binding and specificity information that should be very useful in drug design. Very recently, molecular docking models for cAMP and rolipram, using the PDE4B2B crystal data, were reported [222].

Without any crystal structure, the mutation analysis strategy was assessed to identify amino acids involved in the PDE4 binding activity and selectivity. For example, Asp333 was mutated in HSPDE4D3 in connection with mutations that modify the rolipram sensitivity in RNPDE4B1 [223]. The results showed that Asp333 is involved in the inhibitor binding site. Following a sequence comparison between PDE4 and cGMP-specific PDEs showing that PDE4D3 Asp333 corresponds to a conserved asparagine in cGMP enzymes, the PDE4D3 Asp-Asn mutant was prepared, evaluated and found able to hydrolyse cGMP. In addition, the PDE4D3 Asp-Ala mutant showed a propensity to hydrolyze cGMP. All these data concluded that this Asp residue is also involved in the nucleotide discrimination.

Another approach for designing novel PDE4 inhibitors is to construct a peptidic binding site model using a training set of diverse chemical structures [224], (see diazepino-indoles in chapter of "New Chemical Series in PDE4 Field").

RECENT ADVANCES ON CLINICAL DEVELOPMENT OF THE MAIN SELECTIVE PDE4 INHIBITORS

Rolipram (1) is known as the standard of PDE4 inhibitors. Meiji Seika discontinued rolipram as a nootropic agent during development [225]. Rolipram was licenced from Schering for the potential treatment of depression. Evaluated in phase 2 trials for treatment of tardive diskinesia in Japan, it was also discontinued. Rolipram was investigated for its anti-inflammatory effects in asthma, but was discontinued as well for CNS and cardiovascular side effects [226].

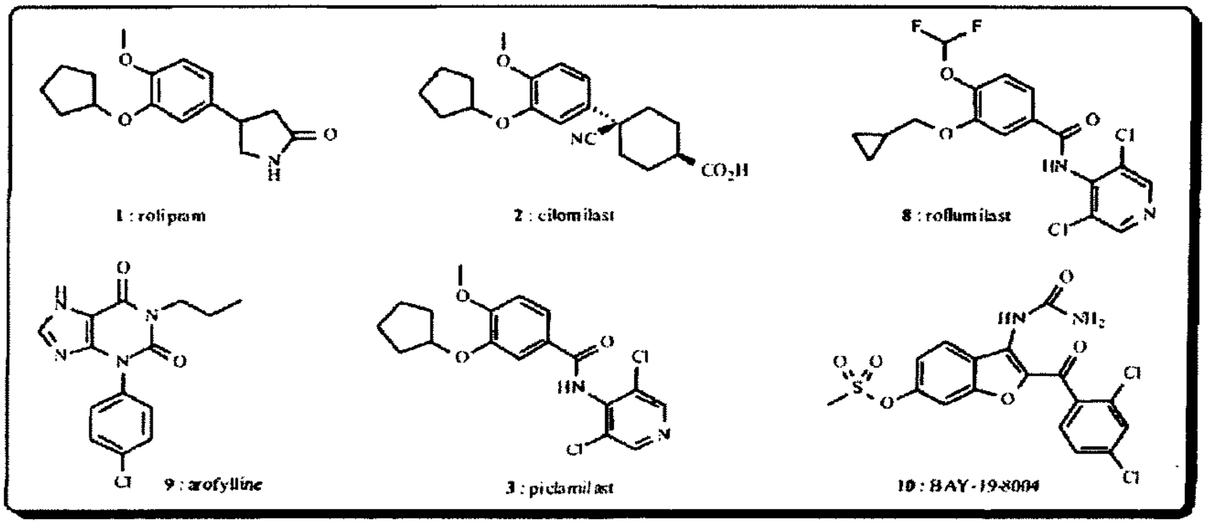
Cilomilast (2, Ariflo®, SB-207499) is developed by Glaxo-SmithKline. Cilomilast is in phase 3 clinical trials as a potential treatment for asthma and chronic obstructive pulmonary disease (COPD). A 12-month, double-blind, placebo-controlled, extension study was conducted to evaluate the long-term safety, tolerability and efficacy of cilomilast in patients with asthma [227-228]. Patients received cilomilast 10 mg BID or placebo for an initial eight weeks when the dose could be increased to 15 mg BID; patients then continued for a further ten months. Clinically relevant improvements above placebo were seen in pulmonary function test parameters from week 1 sustained through month 12. Diary asthma symptoms indicated improvements in cough, wheeze and breath-lessness/chest tightness in the cilomilast group compared to placebo. Cilomilast was associated with small increases in the occurrence of headache, abdominal pain, and nausea. Cilomilast is also in Phase 3 development for the treatment of COPD. Cilomilast 15 mg BID demonstrated improvement in lung function and health status and was safe and well-tolerated during a 6-week phase 2 trial. In this study, cilomilast significantly improved lung function as measured by a 10 percent improvement (130 mL) in FEV_1 from baseline and was generally well tolerated with the most common treatment-related adverse events being nausea, diarrhea and abdominal pain [229]. This first demonstration of clinical efficacy in COPD patients was recently confirmed in a 6-month trial in which patients received cilomilast 15 mg p.o. BID or placebo. This study demonstrated that cilomilast significantly improved health status as characterized by a reduction in the St. George's Respiratory Questionnaire (SGRQ) total score of 4.1 points [230], improved lung function (averaged 40 mL improvement of FEV_1 versus placebo over the duration of treatment [231], and reduced the risk of exacerbation by 39% relative to placebo [232]. Cilomilast was safe and well tolerated in this study; the few gastrointestinal adverse events reported by cilomilast-treated patients were generally self-limited and mild or moderate in intensity [233].

The Byk Gulden PDE4 inhibitor, Roflumilast (8, BY-217), has completed phase 2 asthma studies and is in phase 3 for asthma and COPD. Data has recently been released to indicate that a single dose (1 mg) of the compound inhibits the late asthmatic reaction in bronchial allergen challenge [234], and that 4 weeks treatment with a single oral daily dose (500 μg) inhibits exercise-induced bronchospasm [235]. The first study consisted in a randomized two period crossover study comparing the effects of a single dose of roflumilast versus placebo after allergen challenge. Roflumilast was administered 1 hour prior to the allergen challenge. Mild asthmatics on β_2-agonist treatment only were challenged with allergen using standard protocols. Data of twelve patients who experienced both an early asthmatic reaction (EAR) and late asthmatic reaction (LAR) were analyzed. The maximal LAR to the respective allergen was significantly ($p < 0.05$) inhibited by 62%. Interestingly, there was also a trend in to inhibit the EAR. The second study, a placebo-controlled, randomized, double-blind, two-period, crossover study, was performed to investigate the safety and efficacy of roflumilast in 16 patients with exercise-induced asthma. The patients received placebo and roflumilast (500 μg/day) in random order for 28 days each. In both study periods, exercise challenge was performed 1 h after dosing on days 1, 14 and 28. FEV_1 was measured before the test and repeatedly up to 12 min after the end of exercise challenge. Blood samples for determination of LPS-stimulated TNFα in whole blood *ex vivo* as a surrogate parameter for the inhibition of inflammatory cell activation were taken predose on days 1 and 28. Serial safety measurements were performed during both study periods. Statistical analyses showed a significant superiority of roflumilast over placebo on day 28. The mean percentage fall of FEV_1 after exercise was reduced by 41% as compared to placebo. An improvement of lung function during roflumilast treatment was also observed on days 1 and 14. The median TNFα level decreased by 21% during roflumilast treatment, but remained essentially constant under placebo. Roflumilast was safe and well tolerated during this study. Another study examined the effects of roflumilast on vigilance, visual orientation, motor coordination and concentration. The PDE4 inhibitor was tested at a dose of 500 µg p.o. in the morning for 7 days in a double-blind, randomized, placebo-controlled, crossover study in 18 healthy volunteers. Safety-related performance in traffic and at work was similar on roflumilast and placebo in this trial [236]. A recent publication describes the efficacy of roflumilast in allergic rhinitis [237]. In this randomized, placebo-controlled, double-blinded, crossover study, 25 subjects with histories of allergic rhinitis but asymptomatic at screening received roflumilast (500 µg once daily) and placebo for 9 days each with a washout period of at least 14 days in between treatment periods. In each of the treatment periods, controlled intranasal allergen provocation with pollen extracts was performed daily beginning the third day of treatment, each time approximately 2 hours after study drug administration. Five and 30 minutes after each allergen provocation, rhinal airflow was measured by means of anterior rhinomanometry and the subjective symptoms obstruction, itching, and rhinorrhea were assessed by means of a standardized visual analog scale. Rhinal airflow improved almost consistently during the 9 days of roflumilast treatment, and it was significantly higher at study day 9 on roflumilast in comparison with placebo, a result also found for itching and rhinorrhea. With respect to the subjective obstruction score, a significant difference in comparison with placebo could be demonstrated within 4 days. This study showed that roflumilast effectively controls symptoms of allergic rhinitis and that PDE4 inhibitors might be a future treatment option not only in allergic asthma but also in allergic rhinitis or the combination of the 2 diseases. A 6-month placebo controlled COPD study is currently ongoing but no results have been reported so far.

Arofylline (9, LAS-31025, Almirall Prodesfarma) was initially reported in clinical trials for treatment of asthma by oral route. Arofylline was discontinued at phase 3 in this therapeutic indication. However, in a double-blind, placebo-controlled phase 3 trial in patients with moderate to severe COPD, inhaled arofylline (90 mg daily for three months) significantly showed improvement of lung function (improved FEV1 and reduced incidence of exacerbations). The most common side effect was reported to be transient gastrointestinal effect [238].

The development of piclamilast (3, RP-73401, Novartis, IC_{50} ~1nM)) as an inhaled drug against asthma was discontinued at phase 2 for reason of undesirable side effects and poor pharmacokinetics. Piclamilast was evaluated for the treatment of RA as well. Particularly, RP-73401 displayed a reduction of TNF-α and IL-12 release, an increase of the anti-inflammatory cytokine IL-10, a diminution of T-cell responses and a direct protective effects on bones [239].

BAY-19-8004 (**10**, Bayer) is a benzofuran derivative recently discontinued in clinical phase 2 for the treatment of asthma [240]. The development for COPD has been suspended until all phase 2 findings are complete. With respect to the different PDE4 preparations, BAY-19-8004 provided PDE4 IC_{50}s between 50 and 500 nM [241]. *In vivo*, this compound showed efficacy in various animal models of asthma and COPD. In a LPS-induced lung neutrophilia model in rats, Bay-19-8004 was more efficient than cilomilast (2) in inhibiting the increase of neutrophils in the bronchoalveolar lavage (BAL) with an ED_{50} value at 0.6 mg/kg *p.o.* in comparison to 16 mg/kg *p.o.* [242]. In guinea-pigs, the Bayer compound blocked the ovalbumin-induced eosinophil migration (ED_{50} at 1.5 mg/kg *p.o.*) and reduced the immediate ovalbumin-induced bronchoconstriction (61% at 3 mg/kg *p.o.*) [243]. In the repeated treatment model, BAY-19-8004 (0.1 mg/kg *p.o.* once a day for 10 days) significantly prevented the development of the airway hyperresponsiveness and partially inhibited antigen-induced eosinophilia in BAL [244].



1 : rolipram **2** : cilomilast **8** : roflumilast **9** : arofylline **3** : piclamilast **10** : BAY-19-8004

Finally, conscious ferrets showed no emetic responses at doses reached 30 to 60 fold the efficacy dose [243].

Cipamfylline (**11**, Leo Pharmaceuticals, BRL-61063 or HEP-688) is a selective PDE4 inhibitor currently in phase 2 for the treatment of atopic dermatitis. Previously, SmithKline Beecham discontinued the development of **11** subsequent to phase 1 clinical trials in which doses > 7 µg/kg induced transient nausea, vomiting and headache.

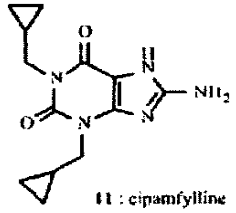
Mesopram (**12**, Schering AG, ZK-117137 or SH-636), a PDE4 inhibitor structurally related to rolipram, is in phase 2 trials for the treatment of relapsing-remitting multiple sclerosis. Recently, the immunomodulatory activity of mesopram was reported in preclinical rodent models. Particularly, this compound selectively inhibited Th1 cell activity with no effects on cytokine production or Th2 cell proliferation [245]. Atizoram (**13**, CP-80633, Pfizer) was originally developed as an oral treatment of asthma, but discontinued in this indication due to emesis in humans at plasma concentration exceeding 0.16 µg/ml [246]. This compound showed efficacy in phase 2 clinical trials for atopic dermatitis [247].

CI-1018 (**14**, Parke Davis, Pfizer), a benzodiazepine compound, showed a good potency and efficacy *in vivo* versus antigen-induced pulmonary eosinophilia model in Brown-Norway rats with an ED_{50} value at 5 mg/kg after oral administration. This compound was not emetic in several species including ferrets: CI-1018 induced no vomiting episodes up to 10 mg/kg *i.p.* whereas rolipram was emetic at 0.1 mg/kg *i.p.* [248-250]. This compound was discontinued at phase 1 for reasons of potential teratogenicity [251]. CI-1044 (**15**, PD-189659, Parke Davis, Pfizer) is a back-up to CI-1018. CI-1044 inhibited TNF-α release from human peripheral blood monocytes and human whole blood *in vitro* with IC_{50} values at 0.34 and 0.84 µM, respectively [252-255]. *In vivo*, this selective PDE4 inhibitor showed higher inhibitory activity than cilomilast in the antigen-induced eosinophil recruitment in Brown-Norway rat with an ED_{50} value at 3.2 mg/kg *p.o.* (cilomilast was inactive at 10 mg/kg *p.o.*). In the LPS-induced TNF-α release model in wistar rats, CI-1044 was equipotent to cilomilast with ED_{50}s at 2.8 and 3 mg/kg *p.o.*, respectively. After a 10 mg/kg intravenous administration, CI-1044 induced no emetic side effects in contrast to cilomilast. CI-1044, after oral administration in rats, was active in inhibiting LPS-induced TNF-α release *ex vivo* [256]. These *ex vivo* data suggested that TNF-α should be used as a marker in clinical studies for evaluating the efficacy of PDE4 inhibitors in blocking inflammatory processes. Very recently, CI-1044 was reported to inhibit plasma TNF-α release in LPS-stimulated whole blood of healthy volunteers and COPD patients. The efficacy of CI-1044 in this assay was slightly better than that of rolipram and much higher than that of cilomilast in the same subjects [257]. These data indicated that TNF-α is an appropriate marker of the anti-inflammatory effects of PDE4 inhibitors in COPD patients and that CI-1044, providing significant anti-inflammatory properties in the context of COPD, may prove efficacious in the treatment of lung inflammation.

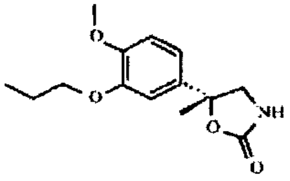
Kyowa Hakko has started phase 1 clinical trials in Europe with the PDE4 inhibitor, KW-4490 (no structure reported), for asthma [258].

CDP-840 (4, Celltech Group / Merck Frost) was discontinued in phase 2 due to unpromising efficacy [146].

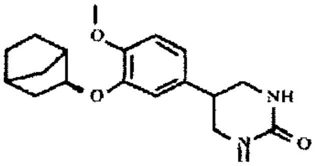
Merck Frosst and Celltech collaborate in the development of a series of PDE4 inhibitors for the potential treatment of asthma, rheumatoid arthritis and COPD [259]. An undisclosed compound was in clinical trials in Europe for the potential treatment of asthma (April 2001). Then, the compound was reported in phase 1 trials (June 2001) and in phase 2 trials (October 2001).



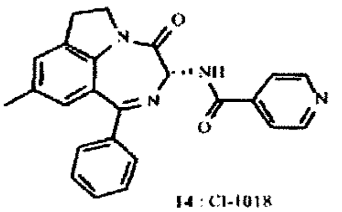
11 : cipamfylline



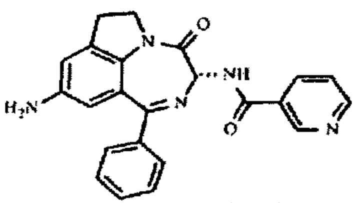
12 : mesopram



13 : atizoram



14 : CI-1018



15 : CI-1044

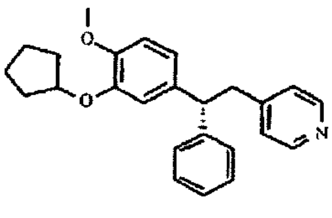
Merck Frost Group has reported data related to L-826,141 (**16**), a new CDP-840 derivative. L-826,141 provided *in vitro* PDE4A activity with an IC_{50} value at 2 nM. *In vitro*, L-826,141 blocked LPS-induced TNF-α release from human whole blood with IC_{50} value at 0.4 μM [260]. Historically, CDP-840 providing short half-lives in various species was further structurally modified in order to reduce the metabolism process. The synthetic efforts led to the identification of a first promising candidate, L-791,943 (**17**), which induced a 96% inhibition in a squirrel-monkey model of ascaris-induced bronchoconstriction after an oral administration of 3 mg/kg [261]. However, L-791,943 showed an excessively long half-life in numerous species. The elaboration of L-826,141, which is an optical isomer of L-791,943, gave an improved pharmacokinetic profile. In the squirrel-monkey antigen-induced bronchoconstriction model, L-826,141 was orally active showing 26-59% and 38-100% inhibition of the early and late phase responses in a dose range between 0.5 and 3 mg/kg [262-263]. In ferrets, L-826,141 was not emetic at 30 mg/kg *p.o.* [261-262]. L-826141 and its predessor, L-791943, were reported to be discontinued as clinical candidates [259].

The development of filamilast (**18**, WAY-PDA-641, Wyeth-Ayerst), a rolipram analog, was discontinued in phase 2.

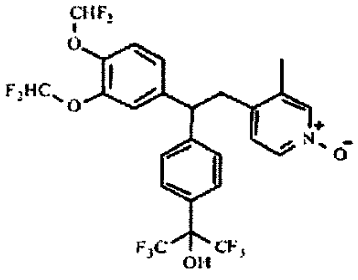
Celltech Group and Schering Plough developed D-4418 (**19**) up to clinical phase 2. D-4418 inhibited human PDE4 activity with an IC_{50} at 200 nM and LPS-induced TNF-α release with an IC_{50} at 160 nM. D-4418 was active *in vivo* in inhibiting TNF-α release in rats (ED_{50} = 10 mg/kg *p.o.*) and in blocking antigen-induced eosinophil accumulation into BAL in guinea-pig (40% inhibition at 10 mg/kg). In terms of safety profile, D-4418 induced no emetic or retching events at 60 mg/kg *p.o.* in ferrets and dogs. In phase I clinical trials, D-4418 showed good plasma exposure and was well tolerated at all doses [264]. Sch-351591 (D-4396, structure not reported) is structurally related to D-4418. This analog, being more potent than D-4418, is under development by Celltech Group (Chiroscience) and Schering Plough for the potential treatment of asthma. Phase I clinical trial has been underway since January 2000 [265].

Icos has recently undertaken a phase I trial with IC-485, a potential oral therapeutic for the treatment of inflammatory conditions [266]. This compound provided an IC_{50} from human recombinant PDE4 of 6.1 nM. The clinical study has been designed to assess the pharmacokinetic properties, safety and tolerability of IC-485 in healthy volunteers and is a double-blind, placebo-controlled, ascending oral dose study.

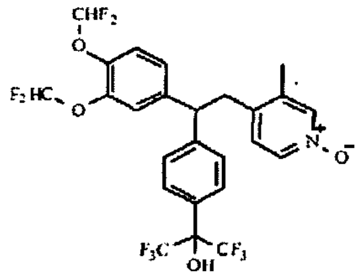
The therapeutic value of YM-976 (**21**, Yamanouchi) was assessed in phase I clinical trials. YM-976 at doses of 0.1 nM to 10 μM showed dose-related relaxation effect on tracheal preparations contracted with histamine and increases cAMP tissue content [267]. A general profile of YM-976 was recently reported [268]. YM-976 was the first compound having shown a dissociation of an anti-inflammatory activity (carrageenan-induced pleurisy) from emetic side effects in the



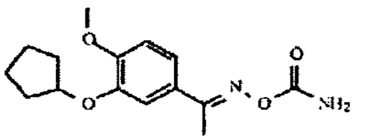
4 : CDP-840



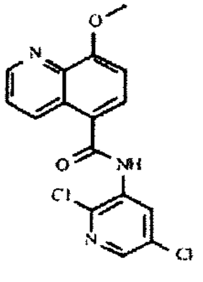
16 : L-791,943



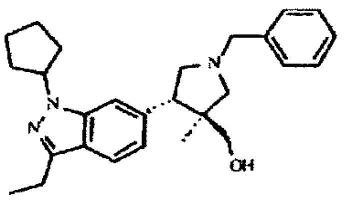
17 : L-826,141 = a L-791,943 optical isomer



18 : filamilast = WAY-PDA-641



19 : D-4418



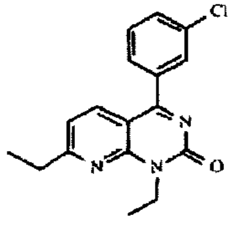
20 : IC-485

same animal species (ferrets). In comparison to various PDE4 inhibitors and particularly cilomilast, YM-976 provided a lower emetic liability and a broader safety profile. The mechanism of the lack of emetic potency remains to be identified. YM-58997 (**22**, Yamanouchi) is investigated under preclinical stage. No development was reported since 1998 for either Yamanouchi compounds.

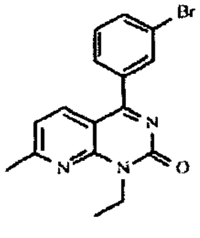
V-11294A (**23**, Napp), a xanthine derivative linked to a rolipram-based structure, entered into phase I clinical trials in 1997. In the ferret emesis model, the compound was safe up to 30 mg/kg *p.o.* keeping into account a bioavailability > 70% and plasma concentrations > 1μM. No emetic side effects were found in phase I clinical trial at doses up to 300 mg with a bioavailability > 50% and a plasma half life > 7 h [269]. Moreover, V-11294A produced a metabolite slightly less potent than itself [156]. In healthy volonteers, a 300 mg oral dose of V-11294A induced a reduction of TNF-α release from a LPS-induced *ex vivo* assay and a decrease of lymphocyte proliferation from a PHA induced model [270-271].

CDC-801 (**25**, Celgene), a thalidomid analog, is a lead of the first generation series of selective cytokine inhibitory drugs (SelCIDs). SelCID compounds are reported as potent inhibitors of TNF-α and PDE4 and presented as potent drugs for the treatment of inflammation, immune disorders, Crohn's disease and congestive heart failure. CDC-801 was evaluated in phase I clinical trials using escalating doses (50 to 1000 mg) and obtained promising results in a phase 2 pilot study for Crohn's disease [272]. In comparison to cilomilast, CDC-801 showed an improved therapeutic index versus emesis [273]. CD-998 (structure not reported, Celgene), a second-generation SelCID, was by far more than 100-fold more potent than CDC-801 against PDE4. CD-998 recently entered in early phase I trials and was found well tolerated. CC-7075 (**26**, Celgene) and CC-7085 (structure not reported), two other second-generation SelCIDs were equipotent and more potent than CDC-801 in inhibiting TNF-α and IFN-Gamma production [274]. CC-7085 was selected as being the back-up of CDC-801 [275].

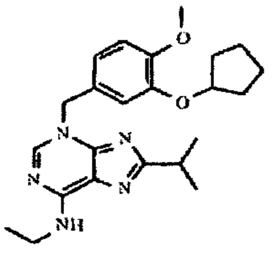
AWD-12-281 (**27**, ASTA Medica Arzneimittel GesmbH) is a 5-hydroxyindole derivative under preclinical investigations for the potential treatment of allergic asthma. Data showed anti-inflammatory effects of the compound with a very low emetic potential [276]. AWD-12-281 provided effect on mucus secretion in the mouse and LPS-induced neutrophilia in the rat and domestic pig [277]. As a model for COPD, the LPS-induced neutrophilia in rats was reduced by 37% and 69% at the dose of 30 mg/kg *p.o.* and 1 μg/kg after intrapulmonary administration [278]. A dose-dependent inhibition of allergen-induced late-phase eosinophilia was shown in sensitized rats (ID_{50} values at 7 μg/kg and 30 mg/kg respectively after intrapulmonary and oral administrations [279]. In a recent paper, RP-73401, rolipram, cilomilast and AWD-12-281 potently were shown to inhibit C5a-induced O_2-generation (IC_{50} = 0.03, 0.42, 0.55 and 0.86 μM, respectively), but failed to inhibit degranulation in the absence of adenylate cyclase activators such as histamine, salbutamol, prostaglandin E2 and forskolin. AWD 12-281 was the only one to block degranulation (IC_{50} = 16.2 μM) without any activation [280]. AWD-12-343 (**28**, ASTA Medica Arzneimittel GesmbH) emerged from a structure optimization process starting from AWD-12-281 and was selected for further preclinical investigations. This compound was potent with a nanomolar *in vitro* PDE4 activity (IC_{50} = 9 nM), highly selective versus PDE3, PDE5, and PDE7 (IC_{50}s > 1 μM) and provided a low affinity for the HARBS (IC_{50} = 313 nM) [281-282]. *In vitro*, AWD-12-343 inhibited TNF-α and GM-CSF release from human inflammatory cells (human polyp cells) with IC_{50} values of 0.22 and 0.26 μM, respectively. In bronchoalveolar lavage fluid (BALF), the



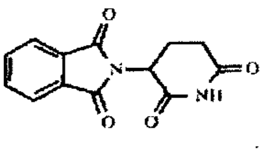
21 : YM-976



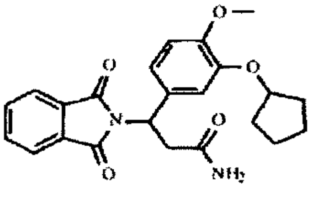
22 : YM-58997



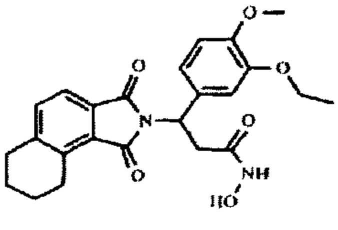
23 : V-11294A



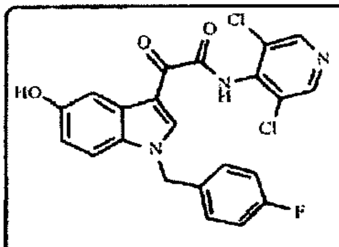
24 : thalidomide



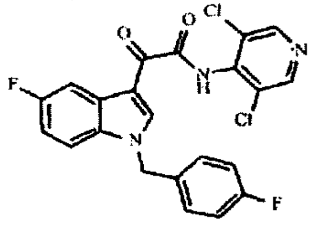
25 : CDC-801



26 : CC-7075



27 : AWD-12-281



28 : AWD-12-343

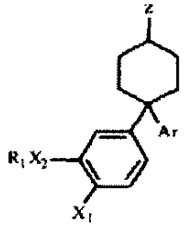
LPS-induced neutrophil recruitment reduced by 37% by AWD-12-281 at 30 mg/kg *p.o.* and by 67%, 61% and 90% after a 1 mg/kg oral administration of cilomilast, rolipram and AWD-12-343, respectively.

NEW CHEMICAL SERIES IN PDE4 AREA

Despite much progress in the development of PDE4, the search for new scaffolds is continuing with the aim to reduce the specific PDE4 side effects. This chapter attempts to give an overview of the global information coming from the scientific literature (papers and patents).

Cyclohexanes, Delta-Lactones or Lactames, Morpholines

A new series of 1,4-substituted cyclohexanes (**29**) structurally related to cilomilast was claimed by SmithKline Beecham (GlaxoSmithKline) [283]. The compounds were found to be PDE4 inhibitors and could be used to treat allergic and inflammatory diseases. They also inhibited the



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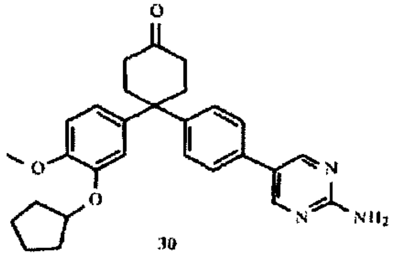

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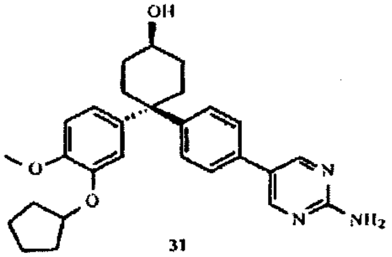

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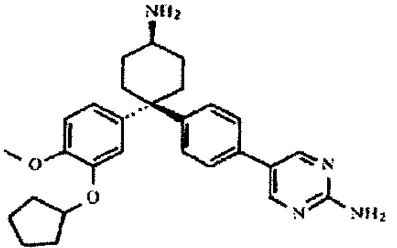

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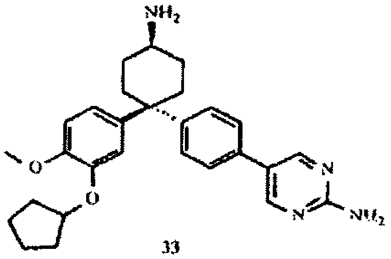

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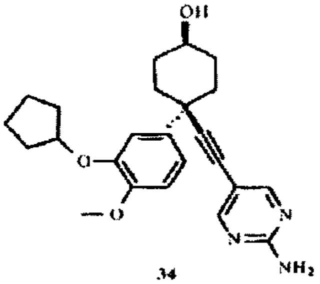


34

production of TNF-α. No biological data were reported and the compounds **30**, **31**, **32** and **33** were shown the most preferred compounds.

A novel construction of the quaternary C4 of such a cyclohexanol ring was reported for the synthesis of a cilomilast derivative, SB-222618 (**34**) [284].

Kyowa Hakko developed new heterocyclic derivatives (**35**) structurally related to cilomilast analogs [285]. (E)-3-[1-(8-Methoxy-2,3-dihydro-benzo[1,4]dioxin-5-yl)-4-oxo-cyclohexyl]-acrylic acid ethyl ester (**36**) inhibited 99% of human PDE4 at 1 μM. In EP-1110961, Kyowa Hakko described *cis*-4-cyano-4-(8-methoxy-1,4-benzodioxan-5-yl)cyclohexane carboxylic acid (**37**) showing 87% inhibition of the PDE4 enzyme at 1 μM [286].

The discovery of a new series of substituted γ-phenyl-Δ-lactones and their analogs including lactames as selective PDE4 inhibitors was described by Inflazyme [287]. (S)-5-(3-cyclopentyloxy-4-methoxy-phenyl)-3-(4-hydroxy-3-methoxy-benzyl)-tetrahydro-pyran-2-one (**38**) provided a PDE4 inhibition from U937 cell line with an IC_{50} value in a range from 0.1 to 1 μM. This compound inhibited the human neutrophil degranulation (via the inhibition of the reactive oxygen species in neutrophils) with an IC_{50} value lower than 1 μM and blocked the TNF-α production in a concanavalin A-stimulated human CD4+ T-cells with an IC_{50} between 2 and 20 μM. In this model, the compound showed an antioxidant activity *in vitro* and a Th1-inhibiting/Th2-sustaining profile. In addition, the compound displayed potent inhibitory activities in the resiniferitoxin-induced mouse ear edema after topical, intraperitoneal and oral administration with 98% inhibition at 50 mg/ear, 72% inhibition at 100 mg/kg and 45% inhibition at 10 mg/kg, respectively.

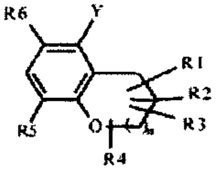
A new series of 2-phenylmorpholine derivatives (**39**) was presented by Nikken Chemicals [288]. Providing strong PDE4 inhibitory activity, these derivatives were reported for the treatment or prevention of inflammatory diseases such as asthma and dermatitis and autoimmune diseases such as multiple sclerosis and rheumatism. No biological data were reported.

Quinolines, Dihydroisoquinolines, Furoisoquinolines, Quinolinones, Phenanthridines, Naphthyridines, Naphthyridinones, Quinazolinones, Pirydopyrazines, Pyridopyrimidines and Pyridylnaphthalenes

In the past, quinoline derivatives were specifically claimed as PDE4 and TNF-α inhibitors by Darwin Discovery [289]. In 2000, the corresponding *N*-oxides (**40**) were described in a new patent [290]. No biological data were reported but these *N*-oxides (**40**), such as the exemplified 8-methoxy-2-trifluoromethylquinoline-5-carboxylic acid (3,5-dimethyl-1-oxypyridin-4-yl) amide (**41**), were stated to be selective PDE4 inhibitors useful for the treatment of inflammatory diseases.

Novel 8-aryl quinoline derivatives (**42**) were recently investigated by Merck Frost for their PDE4 and TNF-α inhibitory activities [291]. The IC_{50} values of the 42 exemplified compounds from human recombinant PDE4A enzyme were stated to be 0.14 nM to 10.2 nM. In an ovalbumin-challenged guinea-pig model, these candidates, at 0.001 to 10 mg/kg *i.p.* or *p.o.* twice a day, induced a significant reduction of the production of eosinophils and leukocytes.

Recently, Sanofi-Synthelabo reported the syntheses of novel isoquinoline derivatives (**44**) as PDE4 inhibitors that could be suitable for the treatment of for instance asthma, COPD and rheumatoid arthritis [292-293]. The PDE4 IC_{50} of isoquinolines **45**, **46** and **47** were in a nanomolar range. The compounds were selective versus PDE2, PDE3, and PDE5.



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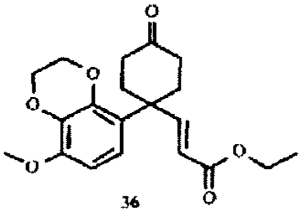

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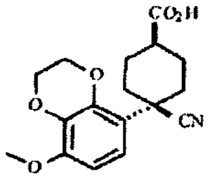

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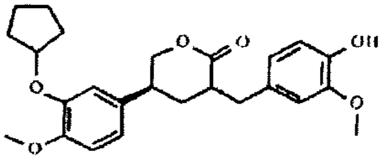

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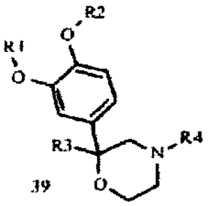

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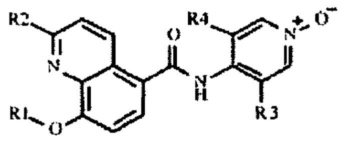

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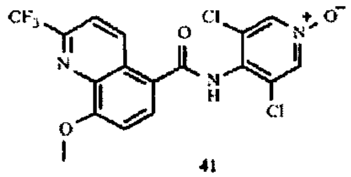

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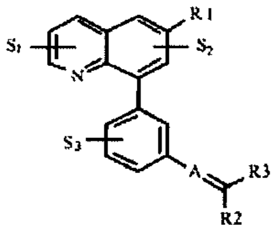
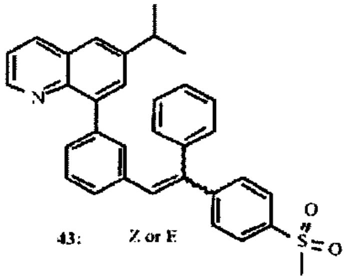
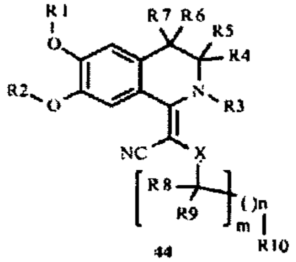
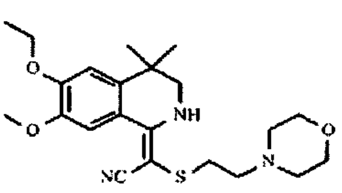


42

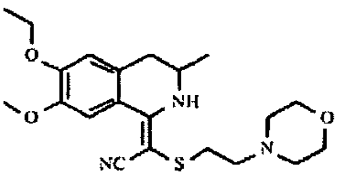




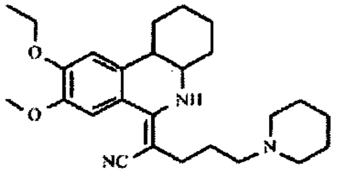
44



45
PDE4 IC$_{50}$ = 2.7 nM



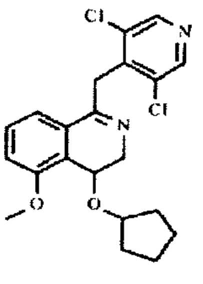
46
PDE4 IC$_{50}$ = 8.8 nM



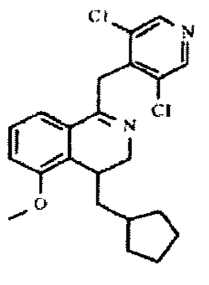
47
PDE4 IC$_{50}$ = 6 nM

In 1999, novel of 3,4-dihydroisoquinolines and 2,3-dihydroquinolin-4-ones were disclosed in Zambon patents in which **48** was described as one of the most potent PDE4 inhibitors *in vitro* with an IC$_{50}$ value at 148 nM [294]. Later on, another series of dihydroisoquinolines was synthesized [295]. The cyclopentylmethylene analog (**49**) of the compound **48** provided an improved *in vitro* PDE4 activity with an IC$_{50}$ at 35.9 nM.

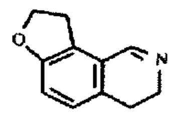
Takeda investigated furoisoquinoline derivatives as PDE4 inhibitors for treating and preventing diseases caused by inflammation [296]. The compounds showing the partial



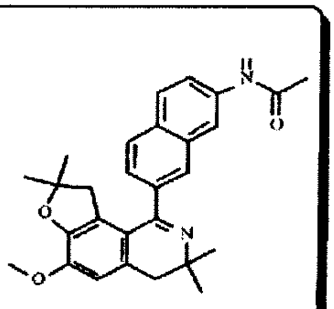
48
PDE4 IC$_{50}$ = 148 nM



49
PDE4 IC$_{50}$ = 35.9 nM



50



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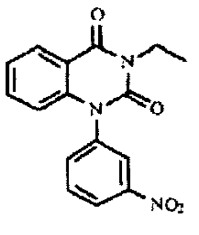
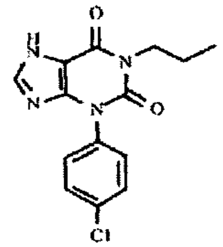
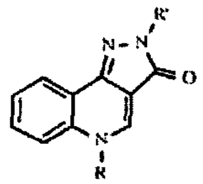


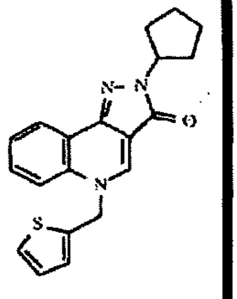
52 - Nitraquazone



9 - Arofylline



53 - DHPQ



54

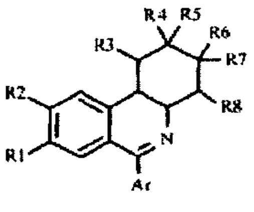
furoisoquinoline structure (50) were disclosed as PDE4 inhibitors. At 1 mg/kg *i.v.*, the compound **51** prevented 59% of constriction in an ovalbumin-induced bronchial constriction model in New Zealand white rabbits.

Almirall Prosdenna group specified a new series of 2,5-dihydropyrazolo[4,3-*c*]quinolin-3-one (**DHPQ**) derivatives as PDE4 inhibitors [297]. These compounds were designed using pharmacophores coming from nitraquizone (**52**) and arofylline (**9**). A lipophilic group at *N2* position was found to be required for PDE4 activity. Compound **54**, with a 2-thienylmethyl group at *N5* position and a cyclopentyl at *N2* position showed a PDE4 activity similar to that of rolipram and an improvement of HARBS/PDE4 ratio > 100 fold. From *in vivo* guinea-pig models, the compound **54** at 10 mg/kg *p.o.* provided 62% inhibition of eosinophil infiltration in BAL and an inhibition of histamine-induced bronchoconstriction with an ED_{50} value at 83 μg/kg after *i.v.* administration. The minimum emetic dose *i.v.* in dogs of that compound was >3 mg/kg.

In 2001, Byk Gulden reported several novel phenanthridine series (**55**) as selective PDE4 inhibitors [298-300]. The *in vitro* PDE4 activities (-logIC_{50} values) of the phenanthridines (**55**) were ranged from 6.76 to 8.87. Among the most potent derivatives, compounds **56**, **57** and **58**, which are *cis*-enantiomers with negative optical rotation data, provided submicromolar IC_{50} values.

Within the same time period, Byk Gulden published three patents related to the PDE4 activity of novel phenanthridine-*N*-oxide derivatives (compounds **59**) [301-303]. The *in vitro* activities (-logIC_{50} values) of compounds **60**, **61** and **62** were 6.73, 6.29, and 6.09 respectively. Again, these compounds were reported to be the (-)-*cis*-enantiomers.

Novartis recently described the synthesis and SAR of 6,8-disubstituted-1,7-naphthyridines (**63**) that are potent PDE4D subtype-selective inhibitors [304]. The most potent compounds (**64-66**) showed activity in an *in vivo* model of allergic asthma. An appropriate substitution at 8-position (NO_2, Cl, CN) was reported to be important for good PDE4A-D inhibitory activity. Then, the compounds with a small group, such as H or NH_2, at 6-position provided a weak micromolar PDE4A-D activity. The introduction of bulkier groups at this position increased the inhibitory potency toward PDE4B and mostly PDE4D. The benzoic acid derivative (**64**) was the most active compound in this series with a PDE4D activity value at 1 nM. This analog was 49-, 68-, and 88-fold more potent on PDE4D than PDE4B, PDE4A and PDE4C, respectively and was 79-fold



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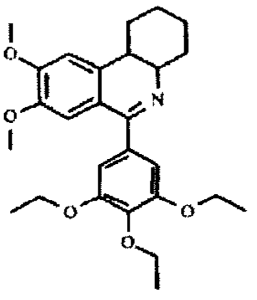
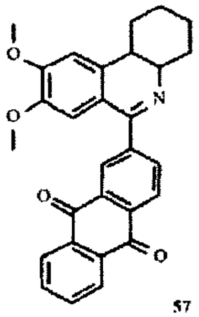
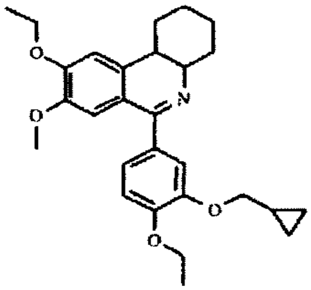


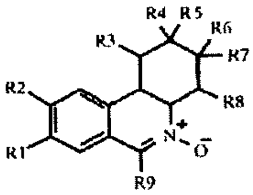
56
-logIC_{50} = 7.81



57
-logIC_{50} = 8.00



58
-logIC_{50} = 8.87



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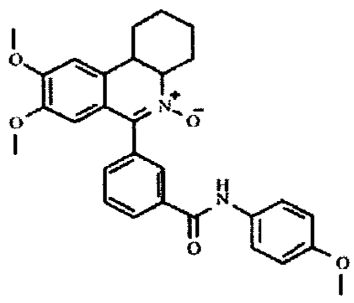
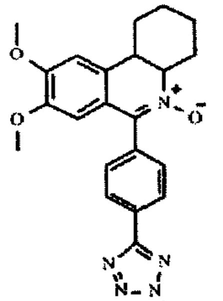
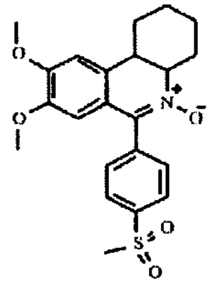


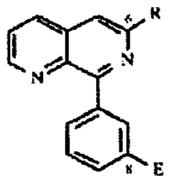
60
PDE4 -logIC$_{50}$ = 6.73



61
PDE4 -logIC$_{50}$ = 6.29



62
PDE4 -logIC$_{50}$ = 6.09



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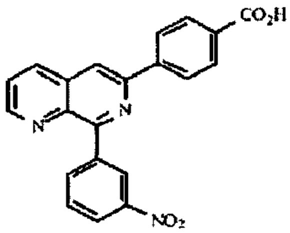

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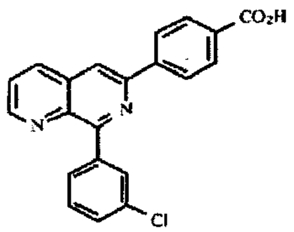

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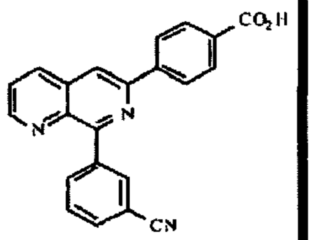
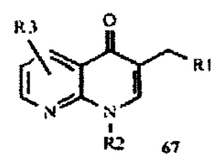


66

more potent on PDE4D than cilomilast. In a model of allergic asthma in Brown-Norway rats, **64** administered at 1 mg/kg *p.o.* decreased by more than 50% the influx of eosinophils, T-cells, and neutrophils in BAL. Finally, at 1 mg/kg *p.o.*, the benzoic acids (**64**, **65** and **66**) were more active than cilomilast in this oral antigen-induced pulmonary eosinophilia rat model.

Pfizer described a new series of 1,8-naphthyridin-4[1*H*]-ones (**67**) [305]. Such derivatives were reported to selectively inhibit the PDE4D isozyme and to block the pulmonary eosinophil infiltration in monkey BAL after subcutaneous administration.

A series of 1,8-naphthyridin-2-one derivatives was identified by Grelan (compounds **70** such as **71**) and Yamanouchi (compounds **72** such as **73**) for providing new PDE4 inhibitors useful for treating inflammatory and autoimmune diseases [306-307]. 1-(3-Nitro-phenyl)-3-(3-pyridin-3-yl-propyl)-1*H*-[1,8] naphthyridin-2-one (**71**) inhibited the reduction in bronchial capacity by 95% at 3 mg/kg after oral administration. 3-[4-(3-Chloro-phenyl)-1-ethyl-7-methyl-2-



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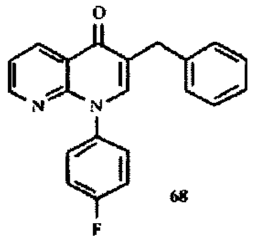

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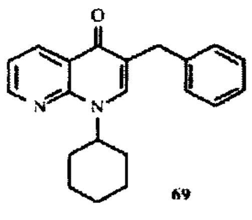

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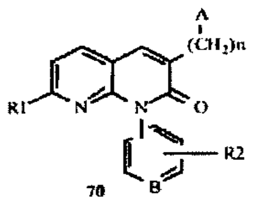

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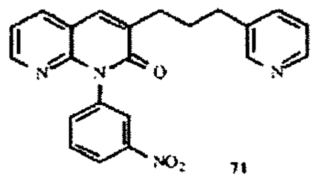

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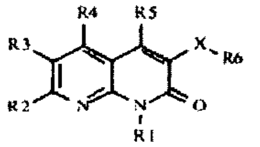

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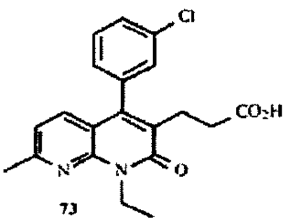

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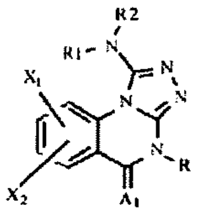

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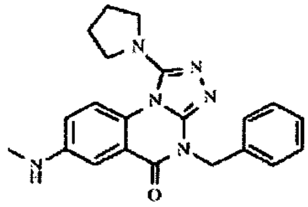
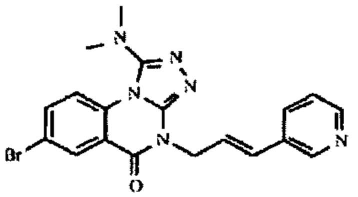
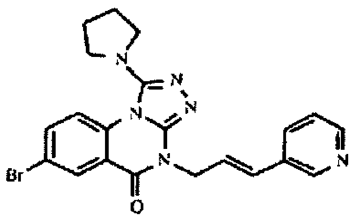


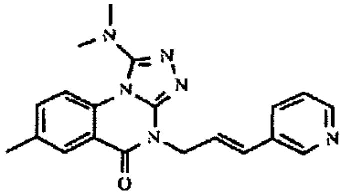
75
PDE4 IC_{50} = 0.00027 μM



77
PDE4 IC_{50} = 0.079 μM



76
PDE4 IC_{50} = 0.014 μM



78
PDE4 IC_{50} = 0.077 μM

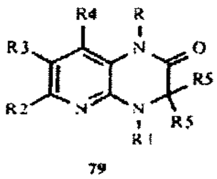
oxo-1, 2-dihydro-[1,8] naphthyridin-3-yl]-propionic acid (**73**) displayed a PDE4 IC_{50} of 0.011 μM.

An extended series of triazolo quinolin-ones or -thiones (**74**) was reported by Parke Davis (Pfizer) [308]. These compounds were found to be selective PDE4 inhibitors. In addition, they inhibited TNF-α release. The compounds provided PDE4 IC_{50}s between 0.00027 and 2.69 μM. Several derivatives inhibited TNF-α release from human leukocytes: the compounds **77** and **78** showed IC_{50}s at 3.4 and 8.1 μM, respectively. Moreover, various compounds were evaluated *in vivo* in a LPS-induced TNF-α model in rats. The compounds **77** and **78** provided 98% and 94% inhibition at the oral dose of 10 mg/kg.

Novel 1,4-disubstituted pyridopyrazine derivatives (**79**) synthesized by Yamanouchi were claimed PDE4 inhibitors [309]. No biological data were presented and the specified compound was 1-(3-bromophenyl)-4-ethyl-6-methylpyrido[2,3,b]pyrazine-2,3(1*H*, 4*H*)-dione (**80**).

In the past, pyridopyrimidine moiety was reported for exhibiting PDE4 inhibitory activity. Several pyridopyrimidine derivatives (**81**) were also disclosed as PDE4 inhibitors by Yamanouchi [310]. These compounds were stated to be of use to treating asthma. No biological data were reported and one compound, 5-(3-bromophenyl)-2-methyl-8,9-dihydroimidazo[1,2-*a*]pyrido[3,2-*e*]pyrimidine (**82**) was specified.

Very recently, the syntheses and *in vitro* evaluation of a new pyridopyrimidine-2,4-dione series (**83**) bearing substituents on the pyridine ring were described [311]. All the C-4 substituted pyridopyrimidine analogs (**85**) provided more potent inhibitory activity than the corresponding C-3 substituted compounds (**84**) except for the benzyl



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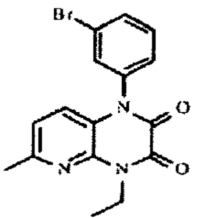

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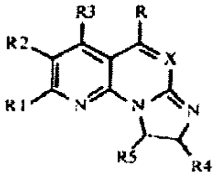

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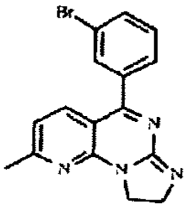

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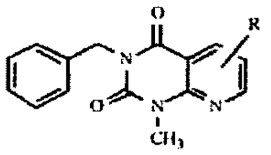

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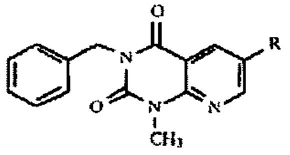
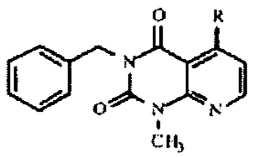


84: C-3 analogs



85 : C-4 analogs

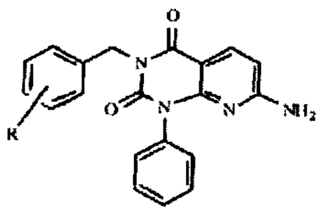
R = O-nBu, CN, CO_2Et, Ph, 4-Cl-Ph, 4-F-Ph, 4-EtO-Ph, 2-F-Ph, 2-CF_3-Ph, 3-NO_2-Ph, 3-CF_3-Ph, CH_2Ph

derivatives. The derivative, with a benzyl group at the C-3 position, was 33 fold more active than its C-4 substituted analog. Moreover, it was equipotent to cilomilast on LPDE4 (IC_{50} at 0.11 μM) and showed a more promising ratio HPDE4/LPDE4 than cilomilast (212.73 instead of 1.64 for cilomilast).

Nippon Zoki also disclosed another series of pyridopyrimidines (**86**) for the treatment of dermatitis [312]. 7-Amino-3-benzyl-1, 2, 3,4-tetrahydro-1-phenylpyrido[2,3-*d*] pyrimidin-2,4-dione (**87**) was the preferred candidate. It was reported to provide a selective PDE4 inhibition versus PDE2, PDE3, and PDE5 with 82% inhibition at 100 μM.

Phthalazines, Phthalazinones, Pyridazones, and Pyridazines

Zambon group prepared some rigid analogs of RP-73401 (**3**) to gather information on the conformation that could be adopted by the phenyl ring in the binding site [313]. Therefore, they designed new cyclic derivatives in which the carbonyl group was substituted by a π-bond involved in a pyridazine nucleus which was also supposed to increase the polarity of the new RP-73401 analogs (**88**). The activities of most of phthalazines (**88**) with PDE4 IC_{50} values < 100 nM showed that, for the RP-73401 related compounds, a planar dihedral angle between the aromatic moiety and the linker was allowed. The phthalazines **88** with a cyclopentyloxy group on R1 were reported to be the preferred compounds in comparison to the derivatives with a cyclopentyloxy on R2. The phthalazine (**89**) displayed a PDE4 IC_{50} value at 53 nM and provided improved safety margins versus acid secretion and emesis against several PDE4 standards. The phthalazine (**89**) diplayed a minimal emetic dose in dogs > 10 mg/kg *i.v.* whereas cilomilast induced emesis through the same route of administration with an ED_{50} at 10 mg/kg. The phthalazine (**90**) was one of the most potent derivative *in vitro* with an IC_{50} value at 8 nM [314]. Based on the decreased potency of the phthalazine (**89**) compared to the open reference compound RP-73401 (PDE4 IC_{50} values at 53 nM and 1 nM, respectively), Zambon group initiated the synthesis of a novel series of phthalazines (**91**) with substituents in position 4 of the phthalazine nucleus [315]. The aim of the study was to avoid a possible unfavorable interaction between the cyclopentyloxy and the *peri-*



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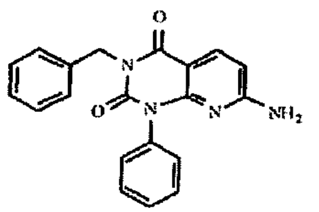

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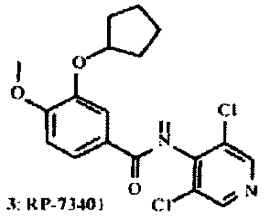
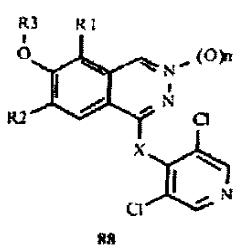


3: RP-73401



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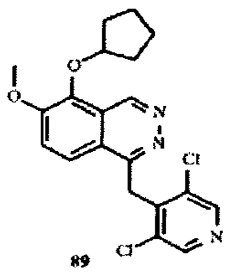

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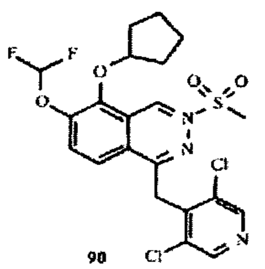

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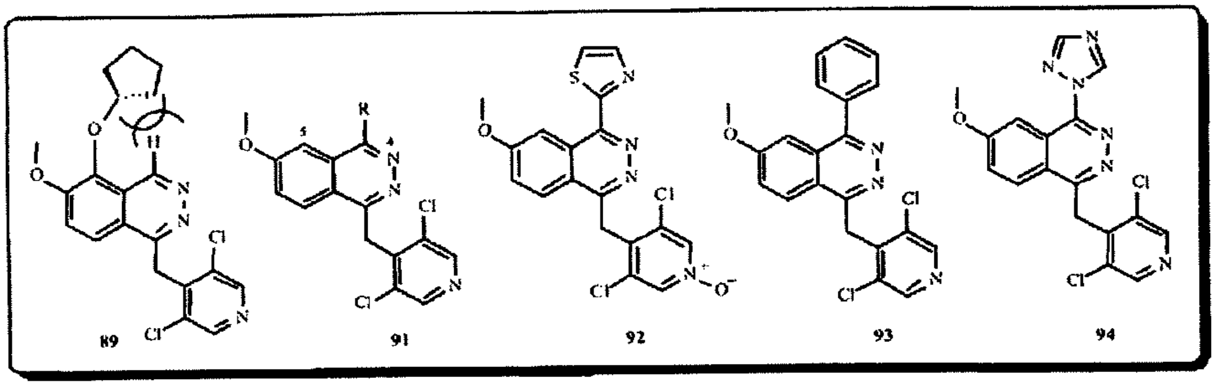


89 91 92 93 94

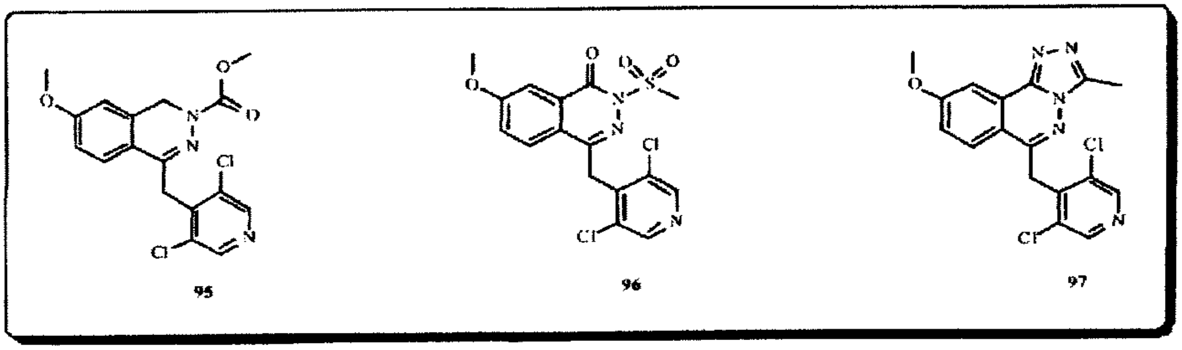
hydrogen in position 4 of the phthalazine nucleus (89) and to evaluate new derivatives (91) with various substituents at position 4 instead of position 5.

Potent PDE4 inhibitory activity was shown with various phthalazines **91**. Only one derivative, the pyridine *N*-oxyde phthalazine **92**, was in a same range of activity than RP-73401 with *in vitro* PDE4 IC_{50} values at 4 nM instead of 1 nM for RP-73401. Most compounds showed *in vitro* PDE4 activities similar to those found with the most active phthalazines **88** (IC_{50}s between 30 and 250 nM). While the structure-activity relationships of phthalazines **91** remained to be explained, several substituents with various physico-chemical parameters led to potent new candidates with potent PDE4 activity and whole cell efficacy. Eosinophilia in guinea-pigs and side effects of two phthalazines, **92** (PDE4 IC_{50} = 37 nM) and **93** (PDE4 IC_{50} = 241 nM) were evaluated. The two compounds were found more active than cilomilast in inhibiting eosinophil infiltration. Finally, acid secretion increase in isolated rat stomach and emesis in dog were reduced in comparison with those found with cilomilast.

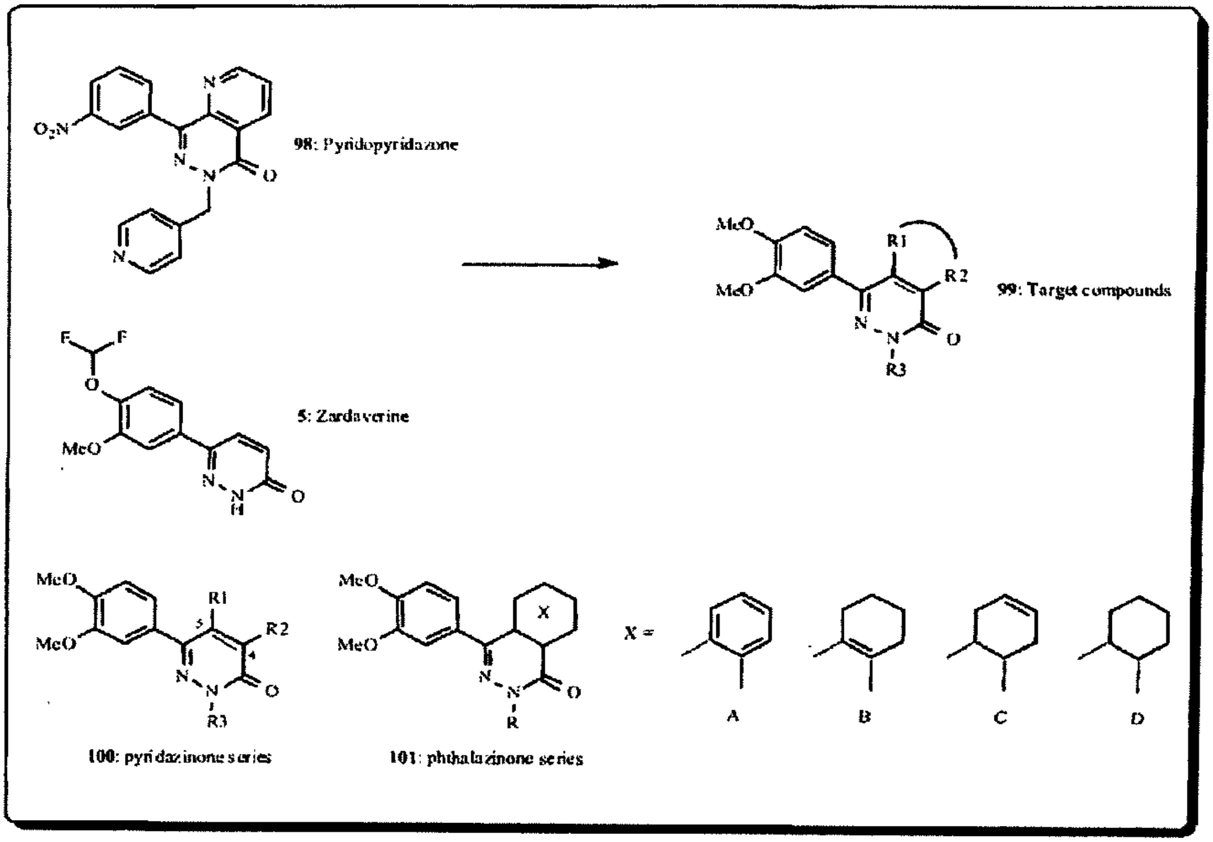
The phthalazine (**95**) with a methoxycarbonyl group and with an *in vitro* PDE4 IC_{50} value at 72 nM was specifically claimed [316]. In addition, several phthalizones were reported: the 3-methylsulphonyl phthalazinone **96** provided a PDE4 IC_{50} at 36 nM.

In a recent patent, Zambon disclosed a series of tricyclic phthalazines and exemplified one compound tested, the tricyclic phthalazine **97** which displayed an *in vitro* PDE4 IC_{50} at 207 nM [317].

Novel PDE4 phthalazinones were recently described by Byk Gulden in two papers. A goal of Byk Gulden was to discover new lead structures (**99**) for selective PDE4 or dual PDE3/4 inhibitors coming from a combination between the pyridazone zardaverine (**5**, a wellknown PDE3/4 inhibitor with a PDE4 pIC_{50} = 6.80 and a PDE3 pIC_{50} = 6.24) and a selective PDE4 pyridopyridazone (**98**, PDE4 pIC_{50} = 8.30) [318]. Therefore SAR studies were applied on a series of 6-(3,4-dimethoxyphenyl)-4,5-dihydro-2*H*-pyridazin-3-ones (**100**) and a series of 4-(3,4-dimethoxyphenyl)-2*H*-phthalazin-1-ones (**101**). The authors described various pyridazinones and a novel series of phthalazinones with potent PDE4 inhibitory potency. Most compounds were found selective PDE4 inhibitors. However, as it was already reported in the literature, SAR studies showed for pyridazinone derivatives with a non substituted amide moiety (R3 = H) the importance of a methyl group at the 5-position for potent PDE3 inhibition. In both series, the *N*-substitution was preferred for a PDE4 activity. The *cis*-4a,5,6,7,8,8a-hexa- and *cis*-4a,5,8,8a-tetrahydrophthalazi-nones (compounds **101** with rings C and D, respectively) were the most potent derivatives with pIC_{50} at 8.4 and 8.1, respectively. Molecular modeling studies showed that the *cis*-fused cyclohex(a)ene ring compounds do not overlap with the other fused-ring phthalazinones: the *cis*-fused cyclohex(a)enes fill a different region of space than the other fused rings which are more or less coplanar with the heterocyclic cycle. Then, the synthesis and structure-activity



95 96 97



98: Pyridopyridazone

99: Target compounds

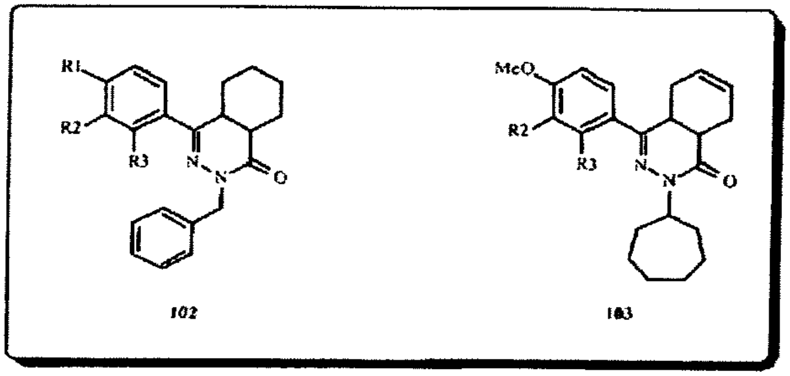
5: Zardaverine

100: pyridazinone series

101: phthalazinone series

relationships of new 4-aryl-substituted *cis*-4a,5,6,7,8,8a-hexa (**102**) and *cis*-4a,5,8,8a-tetrahydrophthalazinone (**103**) series were reported [319]. Several new derivatives were more potent than rolipram and cilomilast in term of *in vitro* PDE4 activity. In both series, the 4-(3,4-dialkoxyphenyl) group was found crucial for potent PDE4 inhibition and the SAR study in this area was generally found similar to those evaluated in the past with 3,4-dialkoxyphenyl moiety of various rolipram-like PDE4 inhibitors such as RP-73401. However, in contrast to the inhibitors structurally related to rolipram, these phthalazinone series were not found affected by the substitution of the 3-methoxy group with larger hydrophobic moieties.

Very recently, Byk Gulden reported novel series of PDE4 phthalazinones (compounds **104** and **111**) [320-321]. In the first patent WO-0130766, the *in vitro* PDE4 activity of 6 *cis*-derivatives (compounds **105-110**) was presented: the -logIC$_{50}$ values were ranged from 7.81 to 9.71. The second patent WO-0130777, was related to new tetrahydrothiopyranyl phthalazinone derivatives (compounds **111**): the -logIC$_{50}$ values of 8 compounds, from 8.02 to



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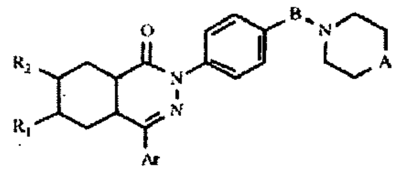

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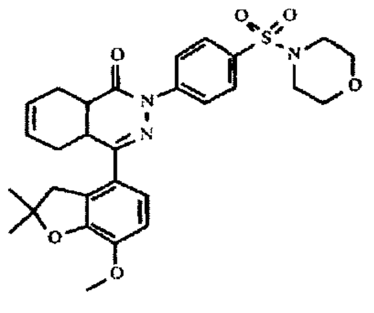
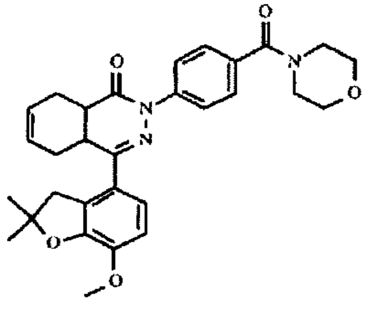
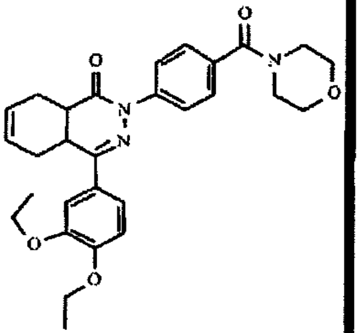


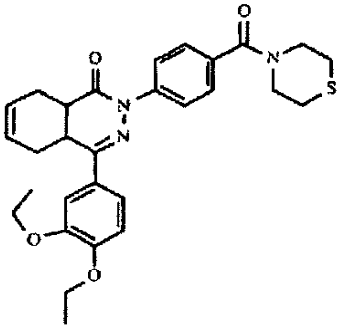
105: PDE4 -logIC$_{50}$ = 9.71



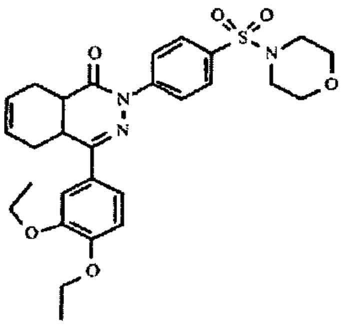
106: PDE4 -logIC$_{50}$ = 8.77



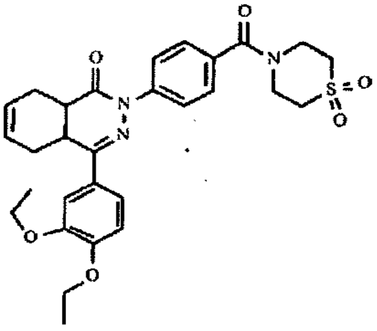
107: PDE4 -logIC$_{50}$ = 7.99



108: PDE4 -logIC$_{50}$ = 7.82



109: PDE4 -logIC$_{50}$ = 9.22

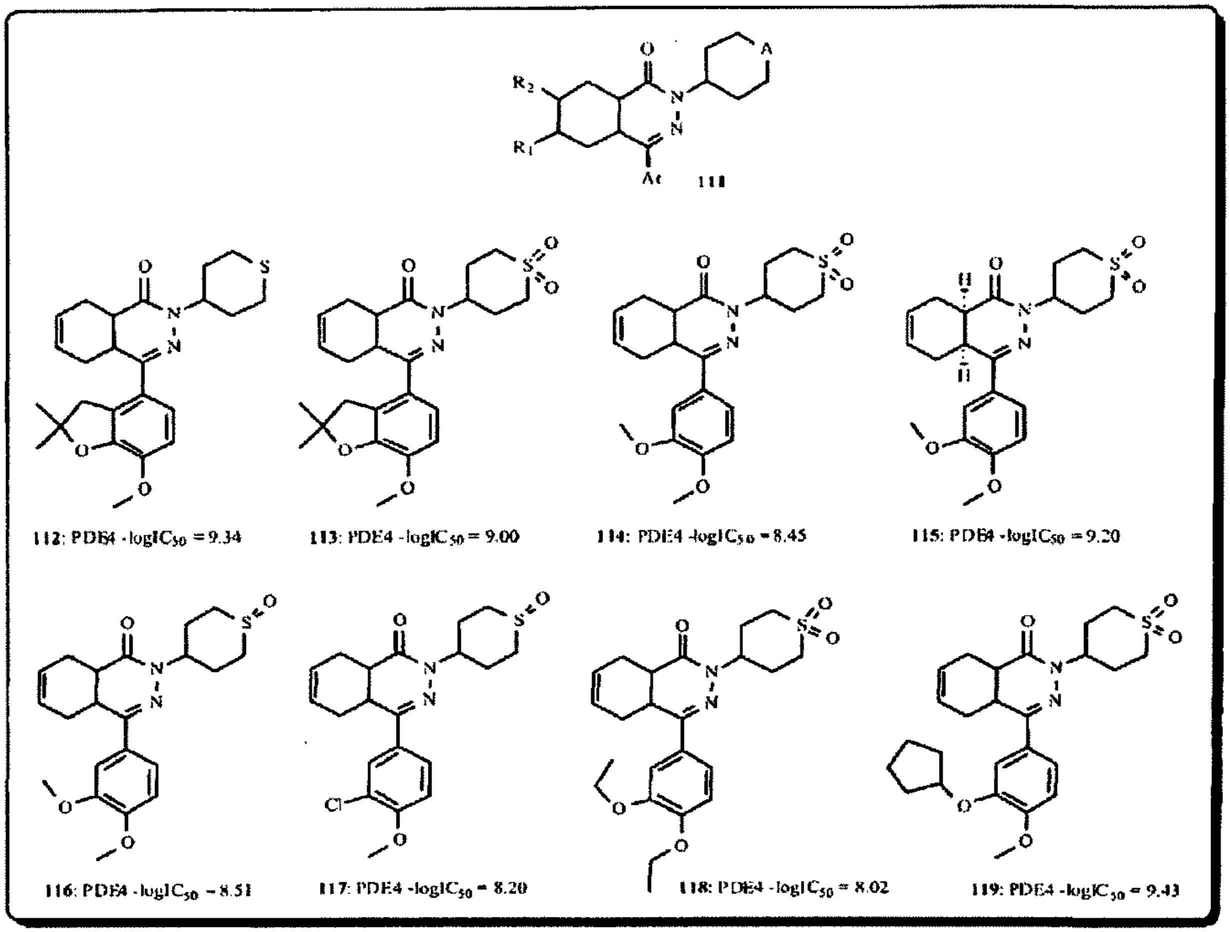


110: PDE4 -logIC$_{50}$ = 7.81

9.43, were reported. The 4S, 8R-*cis* derivatives seemed to be the most potent PDE4 inhibitors (comparison between **114** and **115**). The aryl substitution with the 3-cyclopentyloxy-4-methoxyphenyl group, structurally-related to rolipram, or the 2,2-dimethyl-7-methoxybenzofuran-4-yl moiety increased the *in vitro* PDE4 potency in comparison to the corresponding dimethoxy and diethoxy derivatives (comparison between **119**, **113**, **118** and **114**).

The synthesis of new series of pyridazine derivatives (**120**, **122**, and **123**) were described by E Merck as potent PDE4 inhibitors that could be used to treat allergic, inflammatory and autoimmune diseases [322-326]. No biological data were reported for the new pyridazines (**120**), such as the 4-chloro-*N*-{3-[3-(3-ethoxy-4-methoxy-phenyl)-5,6-dihydro-2*H*-pyridazine-1-carbonyl]-phenyl}-benzamide (**121**), and for the new benzoylpyridazines (**122** and **123**).

In 1996, a new structural class of 1-arylnaphthalene derivatives was reported as showing a selective PDE4 inhibitory activity. Recently, Tanabe designed compounds (**126**) as hybrids of the lead T-440 (**124**) and compound **125** [327]. An efficient synthesis of T-440 was described in 2000 [328]. Among the novel 1-pyridylnaphthlene compounds (**126**), T-2585.HCl (**127**) was a promising PDE4 selective candidate with a PDE4 IC$_{50}$ value at 0.13 nM. This compound provided good antispasmogenic activities in guinea-pig, intravenously for the reduction of the antigen-induced bronchoconstriction (ED$_{50}$ = 0.033 mg/kg) and intraduodenally for the diminution of the histamine-induced



111

112: PDE4 -logIC$_{50}$ = 9.34

113: PDE4 -logIC$_{50}$ = 9.00

114: PDE4 -logIC$_{50}$ = 8.45

115: PDE4 -logIC$_{50}$ = 9.20

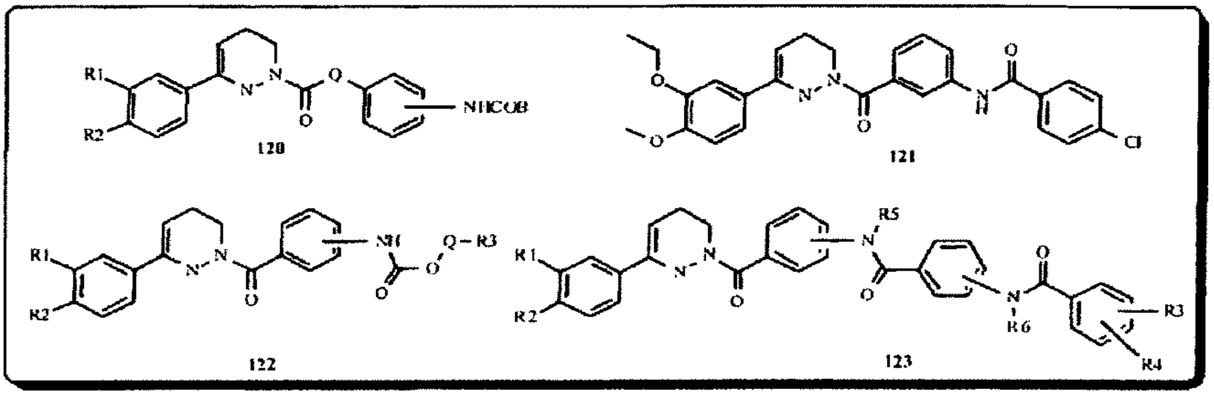
116: PDE4 -logIC$_{50}$ = 8.51

117: PDE4 -logIC$_{50}$ = 8.20

118: PDE4 -logIC$_{50}$ = 8.02

119: PDE4 -logIC$_{50}$ = 9.43

brochoconstriction (ED$_{50}$ = 0.063 mg/kg). Weak cardiovascular effects were observed during the experiments. T-2585 was less emetic than RP-73401 (3) after oral administration in ferrets and intravenous administration in dogs: emetic events appeared in ferrets at 30 mg/kg *p.o.* instead of 1 mg/kg for RP-73401 and in dogs at 1 mg/kg *i.v.* instead of 0.3 mg/kg for RP-73401. In JP-2000063275, Tanabe disclosed 52 new medicinal compounds with a PDE4 inhibiting profile [329]. One of the specified candidate, the 4-(3-hydroxymethyl-6,7-dimethoxyisoquinolin-1-yl)-2-(4-methyl-1-oxo-2*H*-phthalazin-2-yl)pyridine (**128**) displayed 97% inhibition of bronchoconstriction at 1 mg/kg in a guinea-pig histamine-induced asthma model.

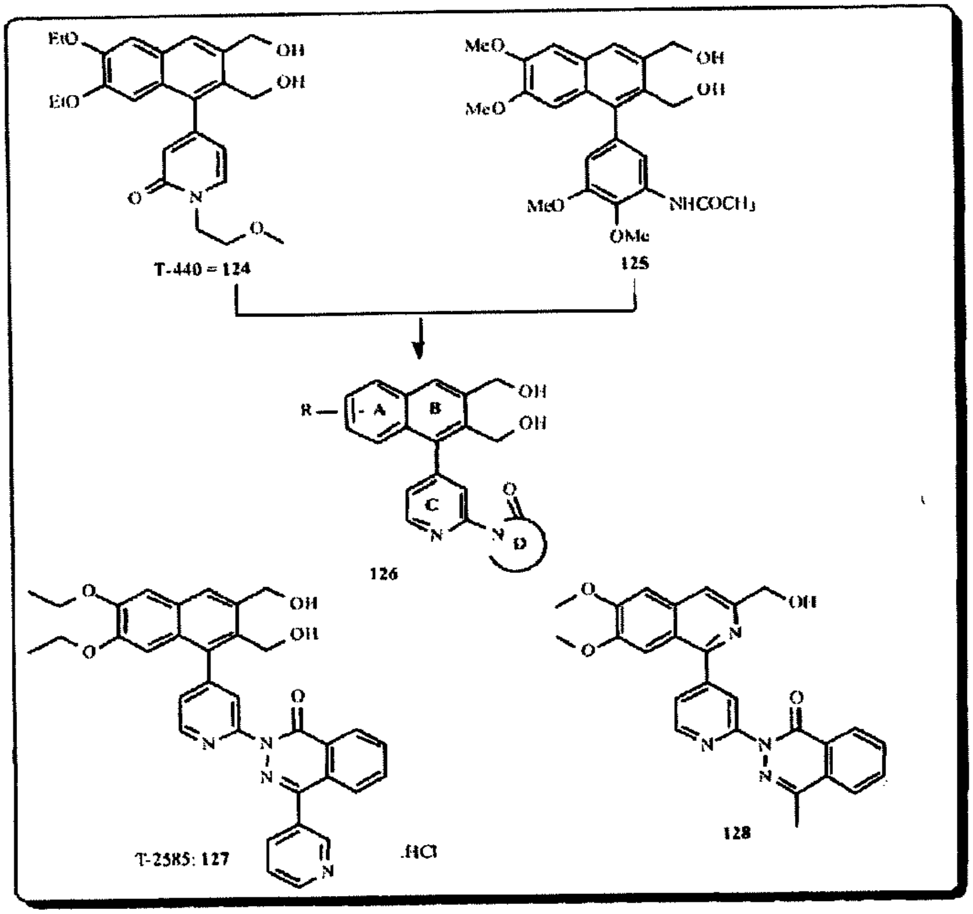


120

121

122

123


T-440 = 124

125

126

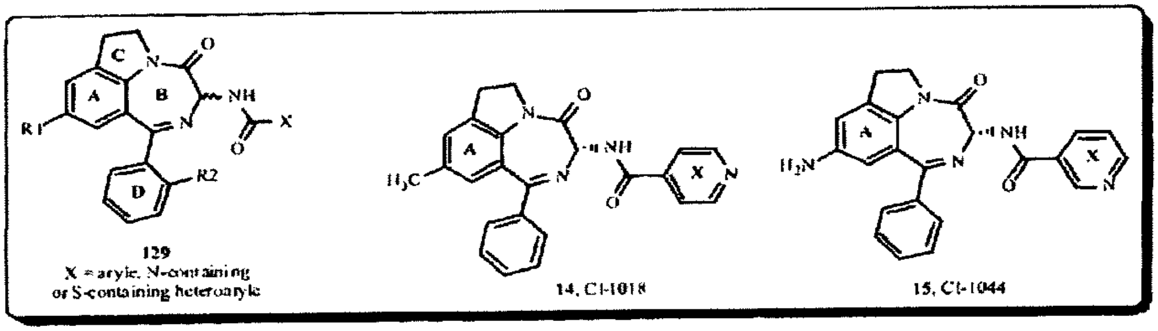
T-2585: 127

128

Diazepinoindoles and Pyrazolodiazepines

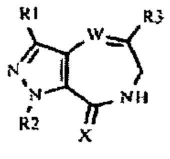
Parke Davis (Pfizer) reported the discovery of a novel series of benzodiazepine derivatives (**129**) as PDE4 inhibitors [330]. Regarding the cycle **C**, the 5-membered ring was preferred for an effective *in vitro* PDE4 activity. In comparison to the derivatives with a 6-membered ring or no rings **C**, the 5-membered ring analogs are supposed to increase the level of structural rigidity and to adopt only 2 low-energy conformations. The active isomer was found to be of R configuration. Classical SAR studies on rings **A**, **D** and **X** were carried out and a single compound, CI-1018 (**14**), was selected for further pharmacological studies [248-251, and see CI-1018 in chapter on clinical development of PDE4 inhibitors].

Later on, starting from CI-1018 (**14**), a SAR optimization was undertaken to evaluate the effects of substitutions on both heteroaromatic rings **A** and **X** and a novel series of aminodiazepinoindoles was described [253]. In comparison to the derivatives with a methyl or a methoxy group at the 9-position (R1), the primary amino analogs

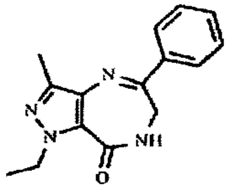

129
X = aryle, N-containing or S-containing heteroaryle

14, CI-1018

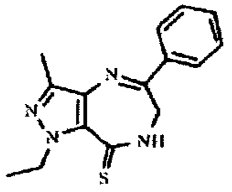
15, CI-1044



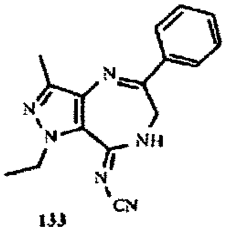
130



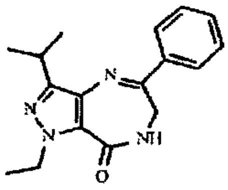
131
PDE4 IC_{50} = 0.088 μM



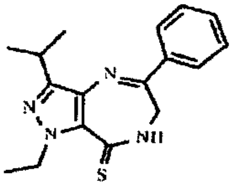
132
PDE4 IC_{50} = 0.06 μM



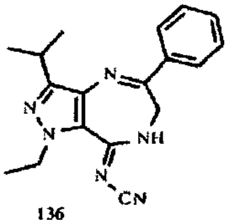
133
PDE4 IC_{50} = 0.0017 μM



134
PDE4 IC_{50} = 0.005 μM



135
PDE4 IC_{50} = 0.001 μM



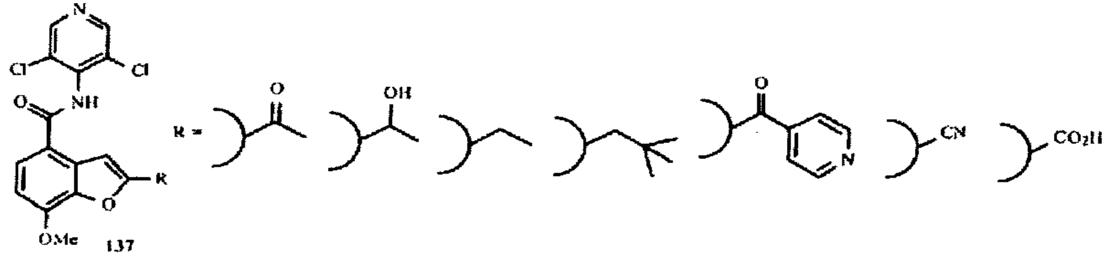
136
PDE4 IC_{50} = 0.00012 μM

were found to be the most potent PDE4 inhibitors with submicromolar PDE4 IC_{50}s. In addition, the NH_2 group was preferred to improve the PDE4 selectivity versus PDE1, PDE3 and PDE5. The mono- and disubstituted amino derivatives reduced the PDE4 selectivity as well. The SAR studies on X-ring remained unclear and several heteroaromatic rings or substituted phenyl moieties were found able to improve *in vitro* PDE4 activity. The improvement of the NH_2 introduction at 9-position was particularly significant through the data obtained *in vitro* (TNF-α inhibition from hWB) but also *in vivo* in rats (inhibition of TNF-α production and BAL eosinophil recruitment). In these different models, most amino derivatives were by far more active than their corresponding methyl analogs and also more potent than cilomilast. Finally, several selected compounds showed improved therapeutic index between efficacy and emesis. CI-1044 (**15**) was selected for further development investigations [254, 256-257, and see CI-1044 in chapter on Clinical Development of PDE4 inhibitors]. The combination of three different techniques of QSAR analysis was applied to the novel series of benzodiazepines not only for improving the understanding of the structural basis for the benzodiazepine affinity to the catalytic center of the PDE4 enzyme but also for providing a new approach in the design of PDE4 inhibitors [331].

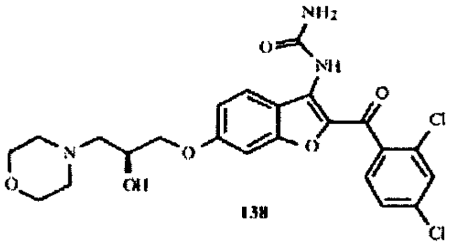
Pfizer recently disclosed a series of novel substituted pyrazolodiazepines (**130**) [332-333]. Several derivatives showed a promising inhibitory potency from human PDE4 enzyme. Some interesting compounds (**131-136**) were from 10 to 7000 fold more active than rolipram (PDE4 IC_{50} = 0.86 μM). The substitution of the oxygen atom of the amide moiety by a sulfur atom led to compounds 1.4 to 5 folds more active. In addition, in the same area, the introduction of a cyanamide group drastically improved the *in vitro* PDE4 activity, ~40 to 50 folds in comparison to the amido derivative activity. It was note worthy that bulkier hydrophobic groups at R1-position increased the *in vitro* PDE4 activity.

Benzofurans, Benzoxazoles, Benzothiazoles, Indoles, Pyrazoles, Pyrrolidines, Pyrrolidinones and Thiazoles

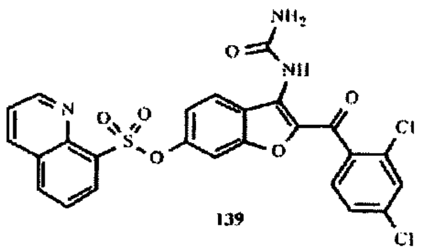
In the past, various benzofuran derivatives were described as potent PDE4 inhibitors. The use of benzofuran nucleus was reported by Rhône-Poulenc Rorer for the replacement of



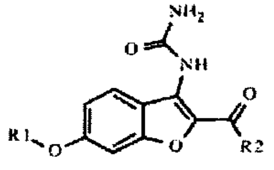
137



138



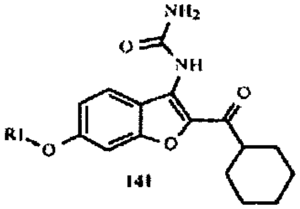
139



140

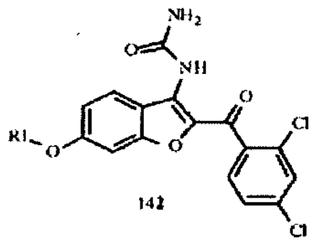
R1 = H, CH_2-Ph, CH_3, SO_2CH_3, CH_2-$C(CH_2)$-CH_2OH, CH_2CHCH_2, $CH_2CH(OH)CH_2OH$

R2 = c-C_3H_5, c-C_4H_7, c-C_5H_9, c-C_7H_{11}



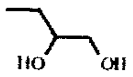
141

the rolipram 3,4-dialkoxy subunit. More recently, Celltech-Chiroscience reported the introduction of new 7-methoxybenzofuran-4-carboxamides with different substituents at the 2-position of the benzofuran nucleus (**137**) [334-335]. Among the compounds **137**, the ketone derivative was found to be the most potent PDE4 inhibitor with an IC_{50} value at 0.0016 μM in comparison to the rolipram IC_{50} at 3.5 μM. This compound provided an important selectivity for the catalytic site versus the rolipram binding site (LARBS/HARBS = 0.037). Then, the replacement of the 3,5-dichloropyrid-4-yl with various 6-membered aromatic rings was evaluated. Only, the 3-chloropyrid-4-yl derivative was in the same range of activity than the corresponding the 3,5-dichloropyrid-4-yl analog but with a lower LARBS/HARBS ratio. Administered at 0.5, 1, and 10 mg/kg *p.o.*, the 2-acetyl-7-methoxy-benzofuran-4-carboxylic acid (3,5-dichloro-pyridin-4-yl)-amide showed good activity across a variety of inflammation-induced

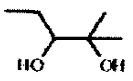


142

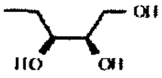
R1 =



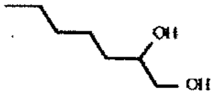
PDE4 IC_{50} = 0.006 μM
O_2-IC_{50} = 0.008 μM



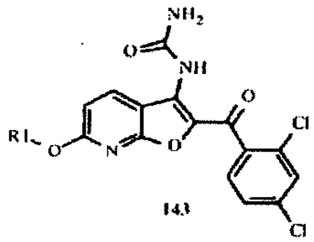
PDE4 IC_{50} = 0.05 μM
O_2-IC_{50} = 0.07 μM



PDE4 IC_{50} = 0.015 μM
O_2-IC_{50} = 0.01 μM

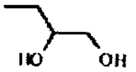


PDE4 IC_{50} = 0.042 μM
O_2-IC_{50} = 0.047 μM

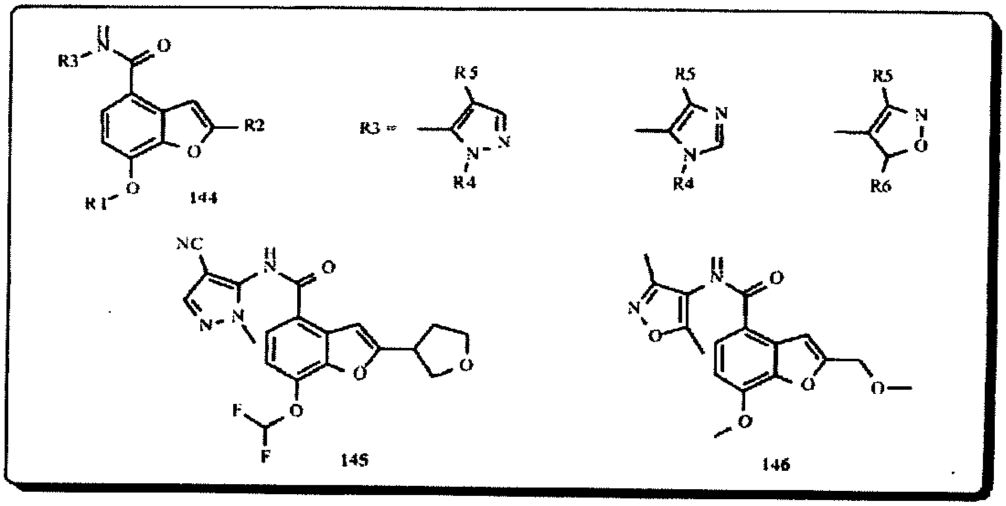


143

R1 =



PDE4 IC_{50} = 0.01 μM
O_2-IC_{50} = 0.008 μM



mediators in a guinea-pig skin eosinophilia model. Emesis and CNS effects were not observed with this compound in ferrets at the oral dose of 10 mg/kg.

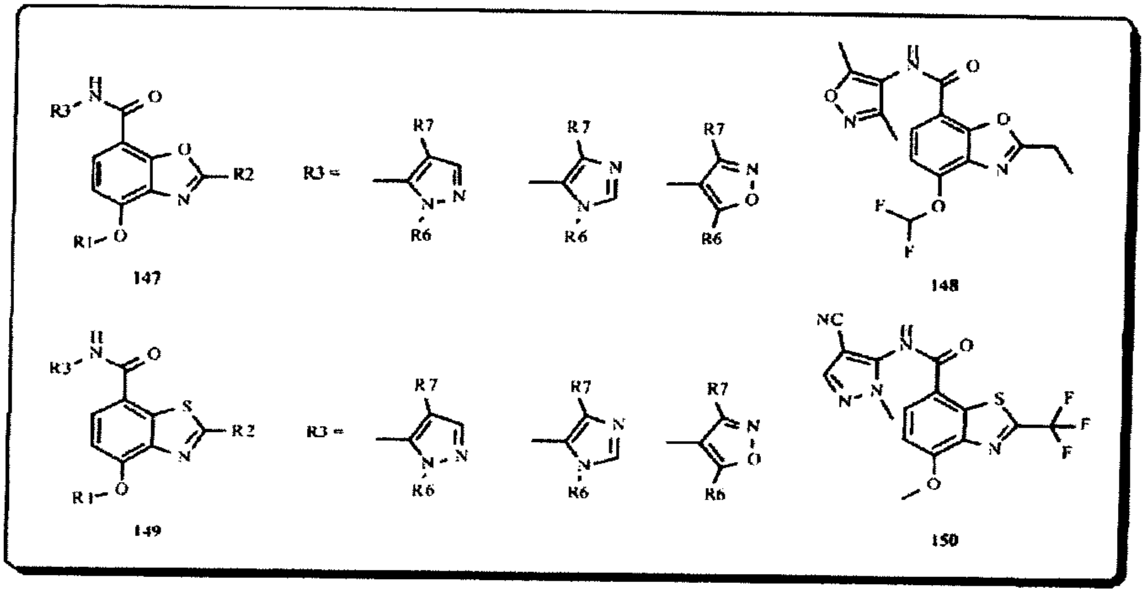
Very recently, Bayer developed new chemical series structurally related to the benzofuran nucleus. Novel benzofuranylaminoalcohols were reported as potent PDE4 inhibitors with HARBS IC_{50}s between 0.01 to 10 µM [336]. The specified candidate (**138**) showed a PDE4 IC_{50} value at 170 nM and inhibited the fMLP-induced superoxide production with an IC_{50} value at 80 nM.

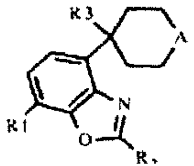
In the same time, a series of new benzofuranylsulfonates was described [337]. One of the most potent compounds (**139**) provided an IC_{50} value at 1 nM in inhibiting PDE4 and in blocking fMLP-induced superoxide production.

Bayer also disclosed a novel series of cycloalkyl substituted 3-urea-benzofuranes (**140**) and of pyridofurane-derivatives [338]. The compounds were reported to inhibit PDE4 activity and fMLP-induced superoxide production with IC_{50} values in a range from 0.001 to 10 µM and from 0.07 to 10µM, respectively. The specified compound was *N*-(2-cyclohexylcarbonyl-6-methoxybenzofuran-3-yl)urea (**141**).

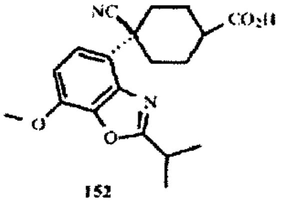
Finally, a new series of oligohydroxyl substituted benzofuran-3-yl (**142**) and pyridofuranylurea derivatives (**143**) was designed [339]. Several compounds were exemplified with their biological data.

New benzofuran carboxamides (**144**) such as **145** and **146** were reported to be TNF-α and PDE4 inhibitors by Darwin Discovery [340]. No biological data were reported.

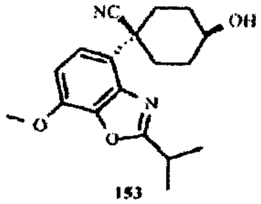




151



152
PDE4 pIC_{50} = 7.69

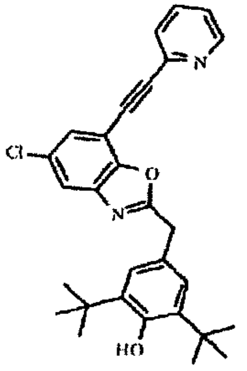


153
PDE4 pIC_{50} = 7.77

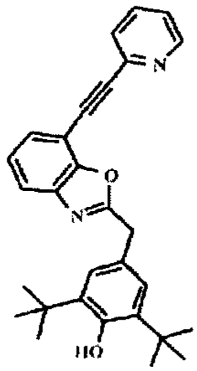
Darwin Discovery investigated novel analog series closely related to these benzofuran carboxamides with the corresponding benzoxazoles (**147**) such as **148** and benzothiazoles (**149**) such as **150** [341-342].

A new class of benzoxazoles (**151**) was disclosed in a patent from Byk Gulden [343]. Most of these compounds might be regarded as hybrid molecules of cilomilast and benzoxazoles. It was reported that the introduction of a hydroxyl (**153**) or carboxylic acid group (**152**) at the 1-position of the cyclo-hexane increased the solubility potency of such compounds. Moreover, the carboxylic acid derivative (**152**) was by far much more active than its corresponding methyl ester analog with a PDE4 pIC_{50}s at 7.69 and 6.46, respectively.

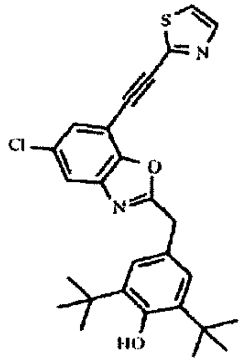
6,5-fused aromatic ring systems including several novel benzoxazole derivatives, acting as selective PDE4 inhibitors, were recently patented by Euroceltique [344]. The compounds, such as compounds **154-157** exhibited higher *in vitro* PDE4 activity and selectivity than rolipram (PDE4 IC_{50} = 3.7 µM) and were also more active to providing bronchodilating and anti-inflammatory effects.



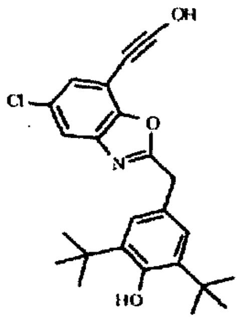
154
PDE4 IC_{50} = 0.013 µM



155
PDE4 IC_{50} = 0.7 µM

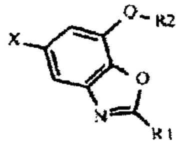


156
PDE4 IC_{50} = 0.014 µM

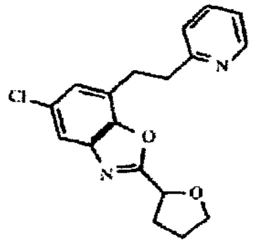


157
PDE4 IC_{50} = 0.42 µM

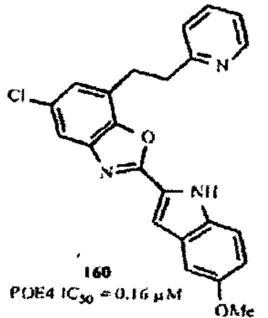
Euroceltique disclosed another new series of benzoxazoles (**158**) [345]. The most potent benzoxazoles were reported to be PDE4 inhibitors with IC_{50}s between 0.16 and 4 µM (in comparison to 3.7 µM for the rolipram). The compounds **159** and **160** were among the most active candidates *in vitro*.



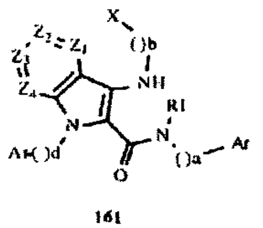
158



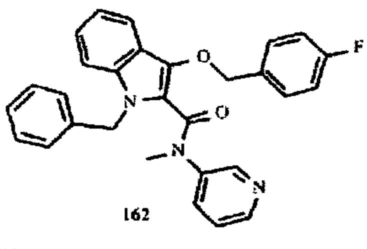
159
PDE4 IC_{50} = 0.19 µM



160
PDE4 IC_{50} = 0.16 µM



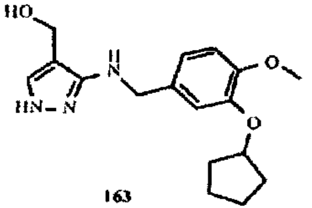
161



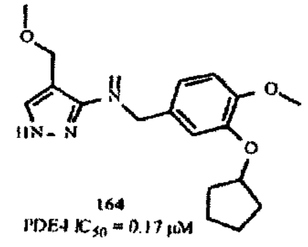
162

The indole-based scaffold was investigated by Merck Frost for the synthesis of new potent PDE4 inhibitors (**161**) such as compound **162** [346]. No biological data were reported.

Euroceltique also reported the synthesis of novel aryl pyrazoles. These derivatives provided similar or improved PDE4 inhibition in comparison to rolipram [347]. The preferred compounds were 3-(3-cyclopentyloxy-4-methoxy-benzylamino)-*1H*-4-hydroxymethyl pyrazole (**163**) and 3-(3-cyclopentyloxy-4-methoxy-benzylamino)-*1H*-4-methoxymethyl pyrazole (**164**) providing PDE4 IC_{50} values at 0.016 and 0.17 μM, respectively in comparison with the rolipram PDE4 IC_{50} at 4.5 μM.



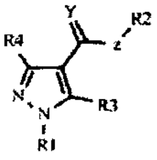
163
PDE4 IC_{50} = 0.016 μM



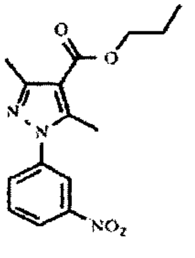
164
PDE4 IC_{50} = 0.17 μM

Novel pyrazoles derivatives (**165**), showing PDE4 and TNF-α inhibitory activities, useful for the treatment of inflammatory disorders were claimed by Icos [348]. The IC_{50} values of 33 compounds were reported between 0.053 and 180.67 μM. Nine compounds showed a LPS-stimulated TNF-α inhibition with IC_{50}s in a range from 0.52 to 6.2 μM. 1-cyclohexyl-3,5-dimethyl-*1H*-pyrazole-4-carboxylic acid ethyl ester (**167**) displayed IC_{50} value of 0.053 μM against human recombinant PDE4B and EC_{50} at 0.52 μM in an inhibition model of LPS-stimulated TNF-α release from human peripheral blood lymphocytes. 3,5-dimethyl-1-(3-nitrophenyl)-*1H*-pyrazole-4-carboxylic acid ethyl ester (**166**) provided a PDE4 inhibition with an IC_{50} value at 0.1 μM and an efficacy *in vivo* in an LPS mouse model with 70% and 100% inhibition respectively at 10 and 100 mg/kg by intraperitoneal injection. The compounds of the invention were also claimed to reduce or eliminate adverse CNS side effects and to produce a minimal or no emetic response.

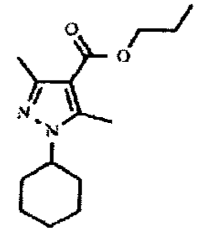
Icos recently claimed new series of pyrrolidine derivatives to be PDE4 inhibitors. New hydrazone and



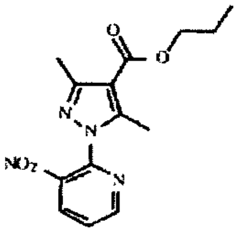
165



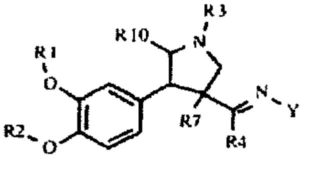
166
hrecPDE4 IC_{50} = 0.1 μM
% TNF-α inhibition: 70% at 10 mg/kg i.p.
and 100% at 100 mg/kg i.p.



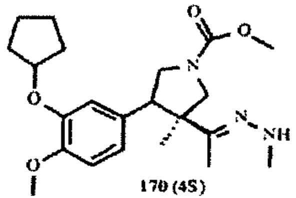
167
hrecPDE4 IC_{50} = 0.053 μM
PBL/TNF-α EC_{50} = 0.52 μM



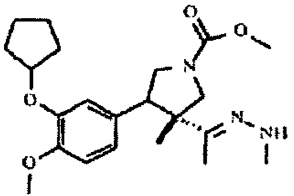
168
hrecPDE4 IC_{50} = 0.2 μM
PBL/TNF-α EC_{50} = 2 μM



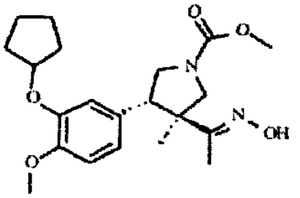
169



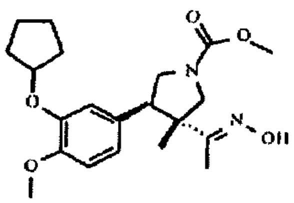
170 (4S)
hrecPDE4 IC_{50} = 115.9 nM
PBL/TNF-α EC_{50} = 76 nM



171 (4R)
hrecPDE4 IC_{50} = 2.2 nM
PBL/TNF-α EC_{50} = 2.2 nM



172 (3S, 4S)
hrecPDE4 IC_{50} = 49 nM
PBL/TNF-α EC_{50} = 281 nM



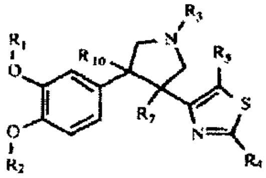
173 (3R, 4R)
hrecPDE4 IC_{50} = 2.2 nM
PBL/TNF-α EC_{50} = 23 nM

oxime analogs of pyrrolidine (**169**) were described in WO-0146136 [349]. The 4 compounds were evaluated using a combined mouse endotoxin-stimulated TNF-α release and locomotor activity assay. The percentages of activity (mobility), at 50 mg/kg *i.p.*, at 64% and 6% for **170** and **171**, respectively, were not related to the *in vitro* PDE4 data. However, accordingly to the *in vitro* PDE4 results, the ED_{50} values for **173** and **172**, respectively, were at 7 mg/kg and > 50 mg/kg.

Novel 3-thiazol-4-ylpyrrolidines (**174**) such as **175** were reported in WO-0146184 [350]. Close analogs (**176**) such as **177** were also reported in WO-0147914 [351].

New pyrrolidines derivatives (**178**) such as **179** were claimed in WO-0147879 [352].

In WO-0147905 and in WO-0147915, Icos disclosed PDE4 pyrrolidines (**180** and **185**) structurally close to the previous series [353-354]. The compounds were claimed for their PDE4 and TNF-α inhibitory potency and for their ability to reduce CNS and emetic side effects. The compounds **181** and **182** provided similar PDE4 and LPS-stimulated TNF-α release inhibition. However, the 2 compounds showed very different behavioral profiles. Therefore, it seemed that the absolute configuration of such PDE4 inhibitors significantly contributed to discriminate



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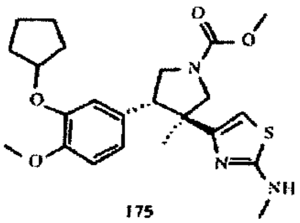
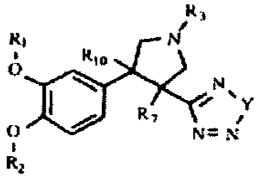


175
hrecPDE4 IC_{50} = 4.73 μM



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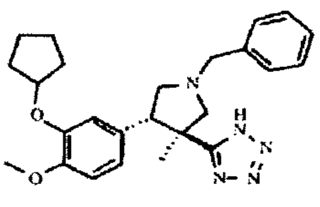
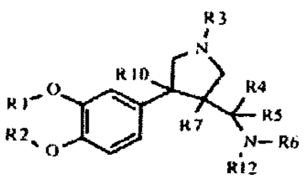
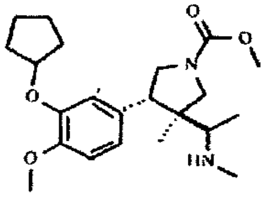


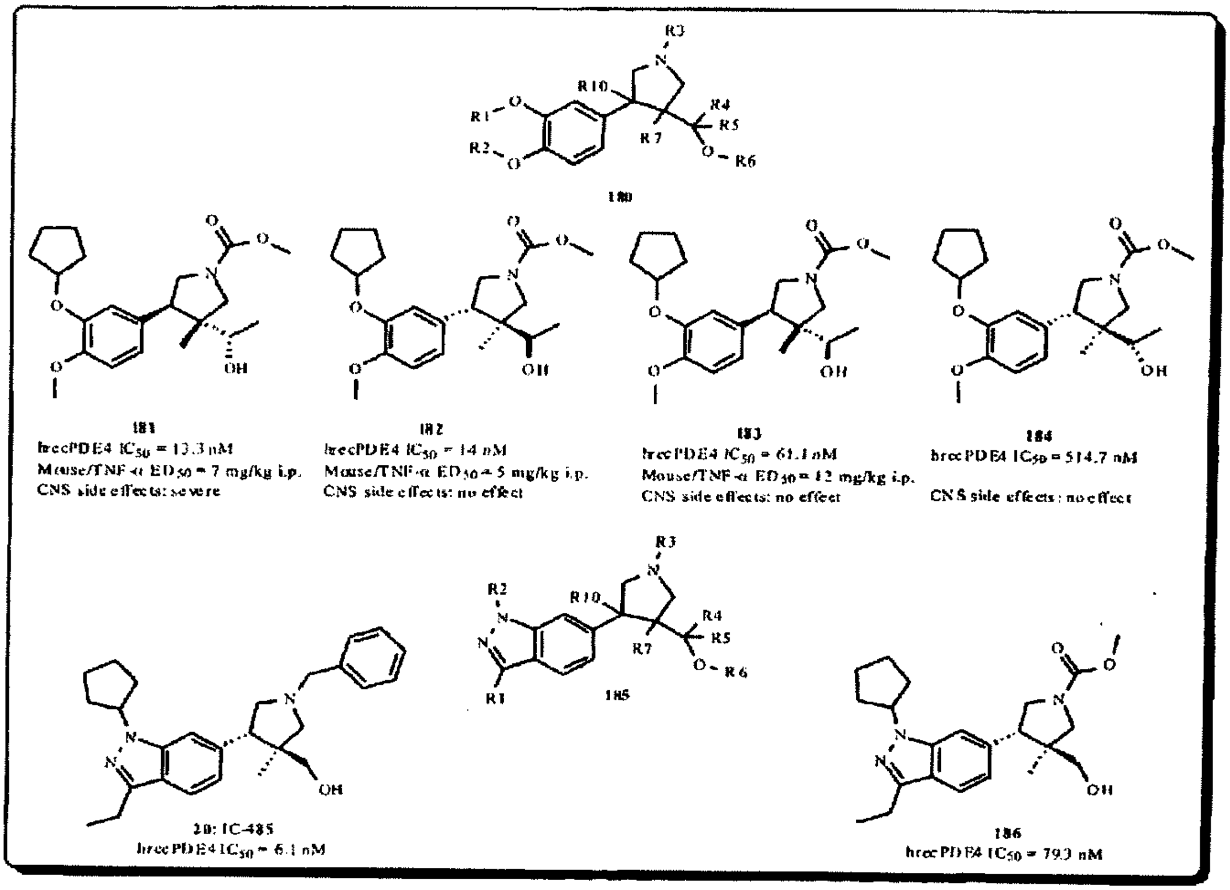
177
hrecPDE4 IC_{50} = 7.9 μM



178



179 = (3S, 4S) mixture of diastereoisomers
hrecPDE4 IC_{50} = 51.7 nM
PBL/TNF-α EC_{50} = 59.0 nM
% mobility at 50 mg/kg i.p. = 145%



180

181
hrecPDE4 IC_{50} = 13.3 nM
Mouse/TNF-α ED_{50} = 7 mg/kg i.p.
CNS side effects: severe

182
hrecPDE4 IC_{50} = 14 nM
Mouse/TNF-α ED_{50} = 5 mg/kg i.p.
CNS side effects: no effect

183
hrecPDE4 IC_{50} = 61.1 nM
Mouse/TNF-α ED_{50} = 12 mg/kg i.p.
CNS side effects: no effect

184
hrecPDE4 IC_{50} = 514.7 nM
CNS side effects: no effect

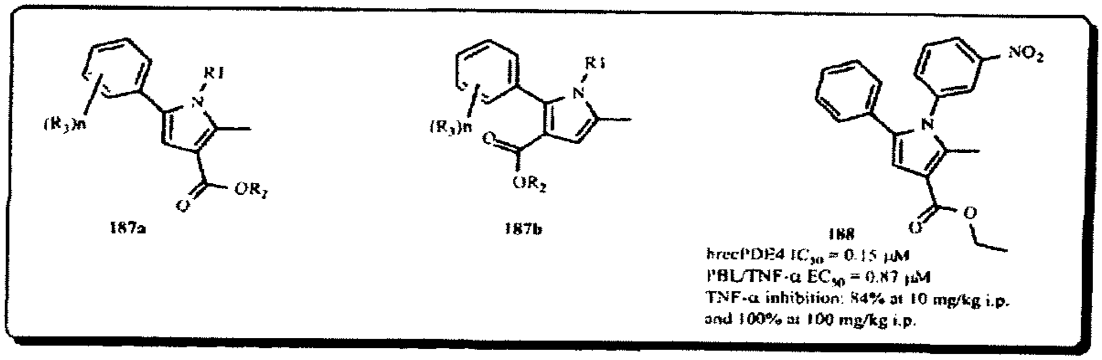
185

20: IC-485
hrecPDE4 IC_{50} = 6.1 nM

186
hrecPDE4 IC_{50} = 79.3 nM

efficacy and CNS side effect propensity. Both compounds **181** and **182**, after oral or intravenous injection (2.5 to 25 mg/kg and 1 to 5 mg/kg, respectively) produced a dose response of the emetic behavior. Compound **181** was by far much more emetic than compound **182**. Recently, IC-485 (**20**), was reported to begin a phase 1 trial for the oral treatment of inflammatory conditions [chapter on clinical candidates, 266].

Icos extended its PDE4 research through the investigation of novel pyrrole derivatives (**187a-b**) [355].



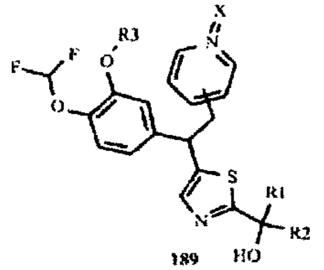
187a

187b

188
hrecPDE4 IC_{50} = 0.15 μM
PBL/TNF-α EC_{50} = 0.87 μM
TNF-α inhibition: 84% at 10 mg/kg i.p. and 100% at 100 mg/kg i.p.

These compounds, such as **188**, were claimed to be PDE4 and TNF-α inhibitors. In addition, they were reported to reduce adverse CNS side effects and to induce a minimal or no emetic responses.

Merck Frost investigated a novel series of thiazole derivatives (**189**) [356]. The candidates were triaryl-substituted ethane analogs. The *in vitro* IC_{50} values with regards to the cAMP hydrolysis inhibition were in a range between 0.01 and 20 μM. *In vivo*, these compounds were active in reducing eosinophilia and accumulation of other



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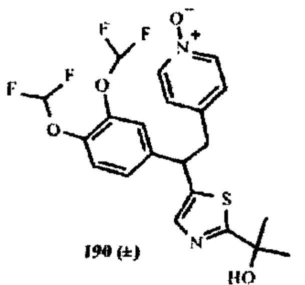
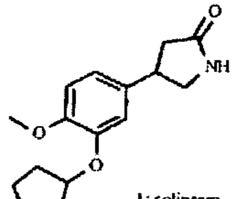


190 (±)

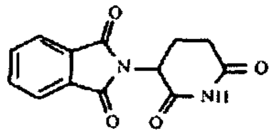
inflammatory leukocytes after oral or intraperitoneal dosages from 0.001 to 10 mg/kg. No specific data were presented.

Isoindolindiones or Thalidomide Analogs

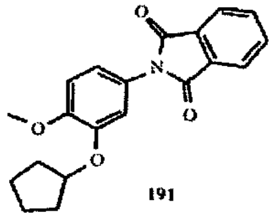
Thalidomide (**24**) was described as a specific inhibitor of a LPS-induced TNF-α production [357]. Therefore, thalidomide analogs were synthesized and demonstrated a good correlation between TNF-α inhibition and PDE4 inhibition. In 2000, Daewoong investigated the TNF-α inhibitory effect of a novel series of compounds designing through a combination between rolipram (**1**) and thalidomide (**24**) [358]. 2 - [3-(cyclopentyloxy)-4-methoxyphenyl]-1,3-isoindolin-dione (**191**) displayed a potent *in vitro* TNF-α inhibition in LPS-stimulated RAW264.7 cells with an IC_{50} at 0.68 μM in comparison to IC_{50}s at 0.1 μM and 194 μM for rolipram (**1**) and thalidomide (**24**), respectively.



1 : rolipram

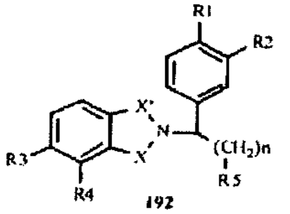


24 : thalidomide



191

In the past, Celgene described modifications of thalidomide derivatives for enhancing TNF-α inhibitory activity. More recently, Celgene synthesized novel thalidomide analogs (**192, 194, and 196**) such as **193, 195** such **197**, respectively. The compounds **192** were found to display multiple potencies including TNF-α antagonism, matrix metalloproteinase inhibition and PDE4 inhibition [359]. The oxadiazoles **194** and substituted acylhydroxamic acids **196** were reported to be PDE4 and TNF-α inhibitors [360-361]. No biological data were reported.



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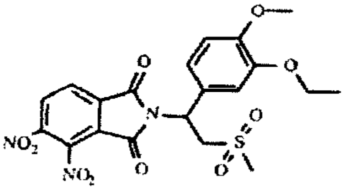

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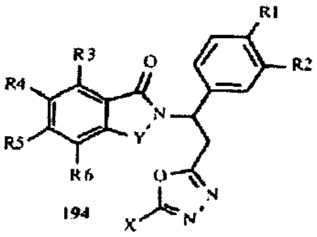
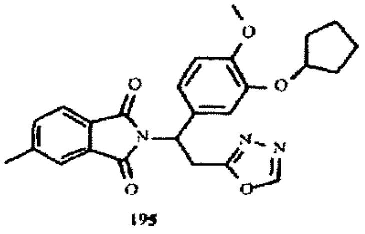


194



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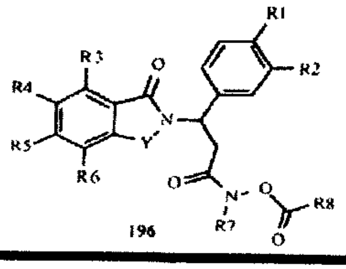
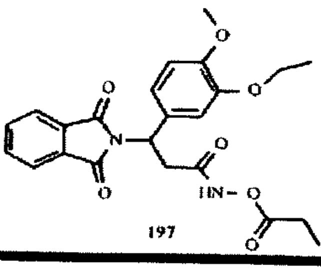


196



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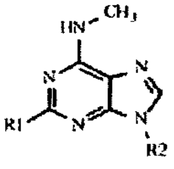

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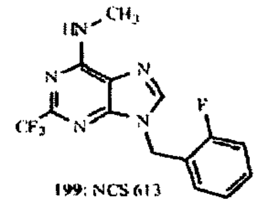
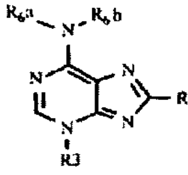
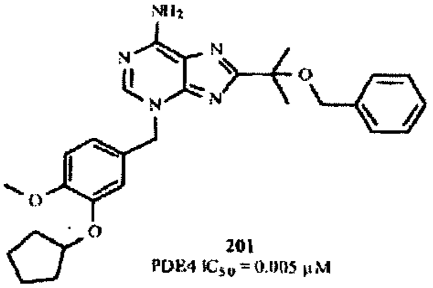


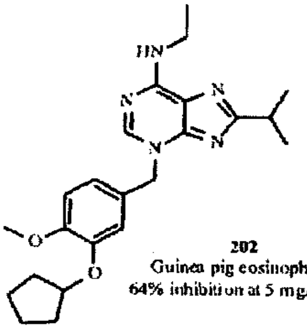
199: NCS 613



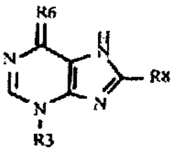
200



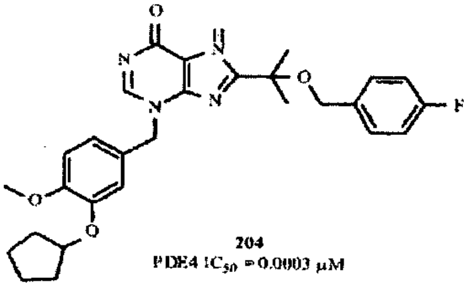
201
PDE4 IC_{50} = 0.005 µM



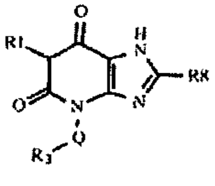
202
Guinea pig eosinophilia:
64% inhibition at 5 mg/kg i.p.



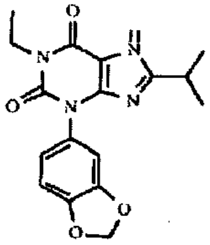
203



204
PDE4 IC_{50} = 0.0003 µM



205

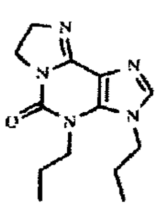


206

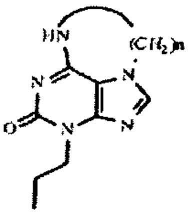
Benzyladenines, Purines and Condensed-Purines

Adenine derivatives substituted at 9-position (**198**) were found to provide a highly selective PDE4 inhibition [362]. These compounds were compared to RP-73401. The most promising compound, NCS-613 (**199**) was further evaluated *in vivo*. NCS-613 (**199**) dose dependently (1, 10, and 30 mg/kg *p.o.*) inhibited the LPS-induced neutrophil recruitment in mice BAL fluid. After intravenous administration of NCS-613 from 0.3 to 30 mg/kg, no gastric acid secretions in rats were observed suggesting that such a compound may produce an improved therapeutic index versus the first-generation of compound (RP-73401).

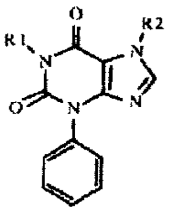
Euroceltique reported new series of purine derivatives (**200**, **203**, and **205**) [363-365]. Such compounds having a PDE4 activity could be useful for the treatment of several diseases including asthma, allergies and inflammation.



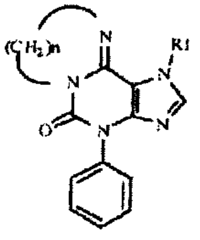
207



208
n = 3
n = 4



209
R1 = nBu, R2 = CH_2COCH_3
R1 = H, R2 = H
R1 = H, R2 = nPr
R1 = nBu, R2 = H



210
n = 2, R1 = nPr
n = 2, R1 = H
n = 3, R1 = nPr
n = 3, R1 = H

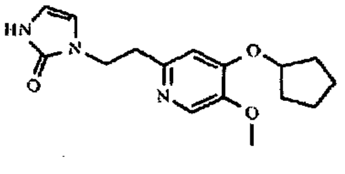
In WO-0059449, 8-(1-Benzyloxy-1-methyl-ethyl)-3-(3-cyclopentyloxy-4-methoxy-benzyl)-3*H*-purin-6-ylamine (**201**) was much more active than the standard theophylline (PDE4 IC_{50} = 1 μM). [3-(3-Cyclopentyloxy-4-methoxy-benzyl)-8-isopropyl-3*H*-purin-6-yl]-ethyl-amine (**202**) was evaluated *in vivo* showing 64% inhibition of eosinophils in guinea pig BAL at the *i.p.* dose of 5 mg/kg. In WO-0111967, a series of hypoxanthine derivatives was investigated. It was stated that such compounds could be potent PDE4 inhibitors, e.g. compound **204** providing a PDE4 IC_{50} value at 0.0003 μM.

In the past, 3,4-dipropyl-4,5,7,8-tetrahydro-3*H*-imidazo [1,2-*i*]purin-5-one (**207**) was described with a selective PDE4 activity. Then, novel condensed-purines (**208**) were designed with the aim of avoiding the xanthime-related adverse effects [366]. The two derivatives with n = 3 and n = 4 were synthesized and found not active on PDE4. Recently, the synthesis of 3-phenylxanthines (**209**) and 4-phenyl[*i*]condensed-purines (**210**) was performed [367]. In both series, compounds showing a PDE4 activity were identified. In the 3-phenylxanthine series (**209**), the derivative with R1 = nBu and R2 = H was the most active on PDE4 with an IC_{50} value at 9.4 μM. In the 4-phenyl[*i*]condensed-purine series (**210**), only the derivatives with R1 = nPr provided a PDE4 activity with IC_{50}s at 15 and 5.7 μM, respectively for n = 2 and n = 3.

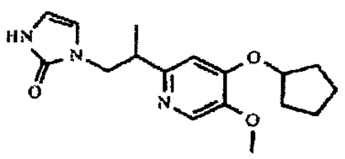
Other Series: Pyridines, Nicotinamides, Benzamides, Arylsulfone Hydroxamates

Janssen described a new series of dialkoxy-pyrydylalkyimidazolidines as selective PDE4 inhibitors [368]. It was reported that a pyridyl group within the catechol pharmacophore might lead to PDE4 active compounds (**211-214**).

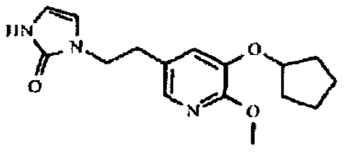
Dainippon disclosed a new class of 2,3-disubstituted pyridines with potent PDE4 activity and good bronchodilating propensity [369]. Therefore, the compounds such as **215** could be used to treat or prevent allergic and inflammatory status. 4-{3-[2-(3-Chloro-phenoxy)-pyridi - 3 - yloxy] - propyl}- pyridin-3-ol (**215**) inhibited guinea pig PDE4 with an IC_{50} at 15.7 nM. *In vivo*, the compound dose-dependently (3-30 mg/kg) inhibited the antigen-induced bronchoconstriction after oral administration.



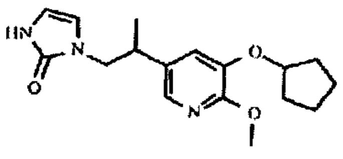
211
PDE4 : 56% inhibition at 1μM



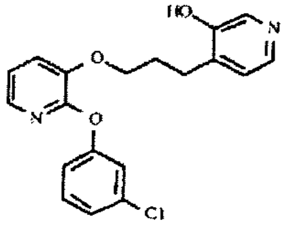
212
PDE4 : 60% inhibition at 100 nM



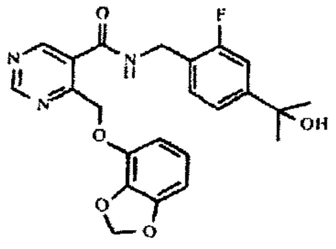
213
PDE4 : 75% inhibition at 100 nM



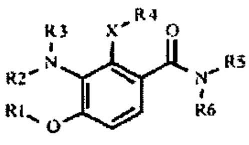
214
PDE4 : 59% inhibition at 100 nM



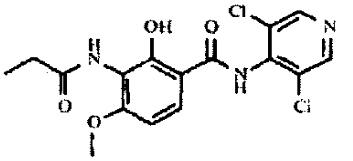
215



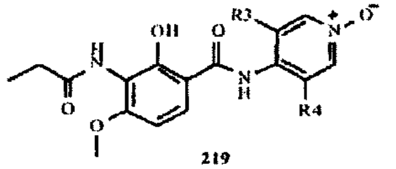
216



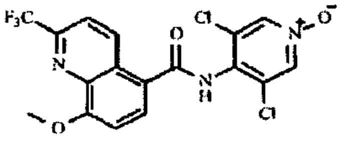
217



218

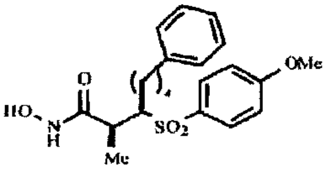


219



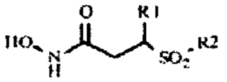
220

New nicotinamide derivatives such as the pyrimidine carboxamide **216** were disclosed as PDE4 inhibitors by Pfizer [370-371]. *In vitro*, the bronchospasmolytic activity was found with IC_{50}s in a range between 0.001 and 1 μM. *In vivo*, the compounds were stated to exhibit in guinea pigs a bronchodilator activity at doses from 0.001 to 0.1 mg/kg i.v. or from 0.1 to 5 mg/kg i.d.: no specific biological data were provided.

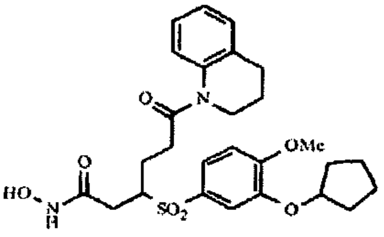


221

PDE4 IC_{50} = 0.03 μM
MMP-1 Ki = 2 μM
MMP-2 Ki = 0.01 μM
MMP-3 Ki = 0.5 μM



222



223

PDE4 IC_{50} = 0.016 μM
MMP-1 IC_{50} > 10 μM
MMP-2 IC_{50} > 10 μM
MMP-3 IC_{50} > 10 μM

In the WO patent applications, WO-0048998 and WO-0026208, novel aminobenzamide and *N*-oxide derivatives (**217** and **219** respectively) were disclosed by Darwin Discovery [372-373]. The compounds **217** and **219** were reported to be selective PDE4 inhibitors and blockers for TNF-α production and eosinophil accumulation. The compounds **219** such as **220** showed improvements with regards to the solubility, the metabolic stability and the pharmacokinetic profile. No biological data were mentioned.

The first compounds simultaneously inhibiting both PDE4 and MMPs 1, 2 and 3 were reported in 1999 by Rhône-Poulenc Rohrer and were represented by the β-(arylsulfonyl)hydroxamic acid (**221**) [374]. A solid-phase synthesis of an arylsulfone hydroxamate lead optimization library (**222**) was further described and the biological activity data directly obtained from the library, without any further purification, showed the potential of this type of the accelerating optimization process [375]. The introduction of a 3,4-dialkoxy group on R2 position provided selective PDE4 inhibitor with a nanomolar range of activity. In order to increase the PDE4 activity, the replacement of the 3,4-dimethoxy-phenyl group by the 3-*O*-cyclopentyl-4-*O*-methtyl-benzene moiety was appropriate in some cases. The modifications of the R1 group showed that steric and polar substituents led to acceptable PDE4 activity.

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Exhibit D

Therapeutic benefit of PDE4 inhibitors in inflammatory diseases

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Intracellular levels of cyclic nucleotides are closely regulated by distinct families of PDEs, which are responsible for the breakdown and degradation of cyclic nucleotides within cells. Type 4 PDEs have the potency to modulate the release of inflammatory mediators through cAMP-dependent and -independent mechanisms. Selective targeting of PDE4 is currently being investigated as a novel therapeutic approach in the treatment of inflammation-associated respiratory diseases such as asthma and COPD. The development of several PDE4 inhibitors, including roflumilast and cilomilast, reflects the success of this approach. In principle, therapeutic intervention of an inflammatory response by PDE4 inhibitors may be extended to other chronic inflammatory disease states such as psoriasis, rheumatoid arthritis and inflammatory bowel diseases (eg, Crohn's disease and ulcerative colitis). This review explores the feasibility of PDE4 inhibitors as a promising alternative for therapeutic intervention in systemic inflammation and inflammation-based disease.

Keywords Arthritis, cAMP, inflammation, inflammatory bowel disease, inhibition, PDE4

Introduction

The second messenger cAMP, generated cytosolically by adenylate cyclases, is pivotal to a myriad of cellular functions. Intracellular alterations in cAMP are translated into cellular responses through cAMP-dependent protein kinase A (PKA). Signaling responses mediated by cAMP are compartmentalized and temporal cAMP gradients allow for the activation of spatially distinct pools of PKA-mediated regulation [1]. Various PKA isoforms are anchored at specific intracellular sites by A-kinase anchoring proteins that allow for discrete PKA populations to respond to cAMP gradients and modify localized target proteins [2,3]. The characteristics of cAMP gradients are dependent upon the activity of adenylate cyclases, which generate cAMP and PDEs that tailor cAMP degradation. The PDE superfamily of enzymes comprises at least 11 members (Table 1) capable of mediating hydrolysis of cyclic monophosphate nucleotides such as cAMP and cGMP [4,5].

PDEs are cyclic nucleotide hydrolyzing enzymes that are spatially distributed in various tissues. While there is a wide distribution of these enzymes inflammatory cells critical to the pathogenesis of inflammatory diseases, including asthma and COPD, preferentially express PDE4. This finding led to the search for subtype-selective inhibitors of PDEs [6-8]. The clinical efficacy of non-selective PDE4 inhibitors (eg, theophylline) provided further evidence for investigating the pharmacological control of intracellular levels of cAMP in proinflammatory and immunocompetent cells [9-11], and for PDE4 as an effective therapeutic target in a variety of inflammation-based diseases. The mechanism of action of PDE4 inhibitors is summarized in Figure 1.

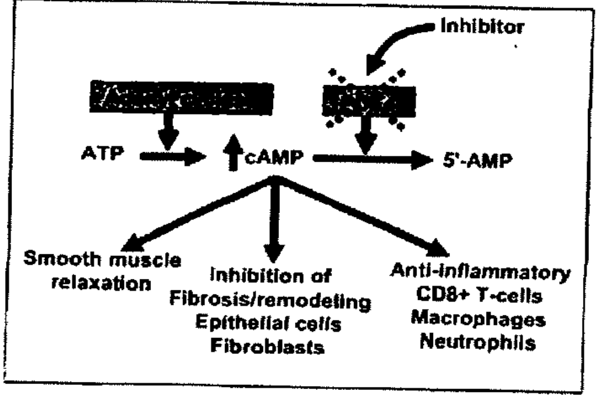


Figure 1. Mechanism of action of PDE4 Inhibitors.

PDE4 subtypes represent the major class of PDEs expressed in a variety of cells, particularly macrophages, eosinophils and neutrophils, which are central to the initiation and progression of an inflammatory immune response. The therapeutic approach is based on the chronic inflammatory nature of certain diseases and the observation that increasing cyclic nucleotides via PDE inhibition leads to the alleviation of various proinflammatory parameters [12,13,14••]. Positive results from pharmacological studies and clinical trials evaluating the efficacy of selective PDE4 inhibitors in respiratory diseases validate this hypothesis [15-17].

Inflammation is the basic response to infection and/or external and internal injury. Both inflammatory and autoimmune diseases are often associated with deregulated expression and biosynthesis of inflammatory cytokines, which influence a plethora of cellular functions. Down-modulation of an inflammatory signal concomitant with amplification of a counter anti-inflammatory signal would provide a rational approach to the treatment of inflammatory diseases. Insights into the molecular mechanisms of the regulatory role of cytokines in the amplification of an inflammatory cascade may also provide a new approach for pharmacological intervention.

PDE4 inhibition and inflammation

The chemotherapeutic potential of PDE4 inhibition is centered on the immunomodulatory properties of cyclic nucleotides. Nonspecific inhibition of PDEs by pentoxifylline results in a pharmacologically efficient modulation of immune functions and has clinical implications in regulating proinflammatory cytokines [18], and the treatment of bronchopulmonary dysplasia [19] and

Table 1. Classification of the PDE family.

PDE family	Substrate	Specific inhibitors	Property
PDE1	cAMP, cGMP	Nimodipine	Ca-calmodulin activated
PDE2	cAMP, cGMP	EHNA	cGMP activated
PDE3	cAMP, cGMP	Cilostamide, milrinone	cGMP inhibited
PDE4	cAMP	Rolipram, Ro-20-1724, Roflumilast (ALTANA Pharma AG/ Tanabe Seiyaku Co Ltd)	cGMP insensitive
PDE5	cGMP	Zaprinast, DMPPO, E-4021, sildenafil	PKA/PKG phosphorylated
PDE6	cGMP	Zaprinast, DMPPO, E-4021, sildenafil	Transducin activated
PDE7	cAMP	BRL-50481 (GlaxoSmithKline plc)	Rolipram insensitive
PDE8	cAMP	Unknown	Rolipram insensitive IBMX insensitive
PDE9	cGMP	BAY-736691	IBMX insensitive
PDE10	cAMP, cGMP	Unknown	Unknown
PDE11	cAMP, cGMP	BAY-656207	Unknown

DMPPO 1,3-dimethyl-6-(2-propoxy-5-methanesulfonylamidophenyl)pyrazol [3,4*d*]-pyrimidin-4-(5*H*)-one, **EHNA** erythro-9-(2-hydroxy-3-nonyl)adenine, ***IBMX*** 3-isobutyl-1-methylxanthine, **PK** protein kinase.

sepsis [20]. cAMP elevation in immunocompetent cells suppresses or inhibits the release of inflammatory mediators and relevant cytokines, as well as the recruitment and activation of inflammatory cells. Cytokines such as IL-1β and TNFα upregulate cell adhesion molecules such as P-selectin, E-selectin, ICAM-1 and vascular cell adhesion molecule [21,22]. PDE4 inhibitors abrogate several T-lymphocyte functions, and intercept T-cell activation and downstream signaling events. Agonists operating via the adenylate cyclase pathway or cell permeable cAMP analogs, inhibit T-cell activation by interfering with phosphatidylinositol turnover, which increases intracellular Ca^{2+} mobilization and protein kinase C activation [23-26]. Effects of the T-cell antigen-receptor (TCR) complex and transcription factors such as nuclear factor of activated T-cells, NFκB and activator protein-1 on phosphorylation have also been reported [27]. Inhibitory effects of cAMP on T-cell functions have been related to PKA-triggered phosphorylation and subsequent activation of the small tyrosine kinase Csk that inhibits Lck, a pivotal tyrosine kinase mediating TCR signaling [28,29]. The localization of PDE4 within the lipid raft signal following anti-CD28 costimulation may also form the basis for the efficacy of PDE4 inhibitors in preventing multiple T-cell functions triggered by anti-CD3/anti-CD28 costimulation [30]. Thus, the mechanics of inhibition are pertinent to allergen-induced T-cell activation and therefore of consequence to allergic disease. PDE4 inhibitors suppress the production of proliferation-driven Th1 and Th2 cytokines such as IFNγ, IL-2, IL-4, IL-5 and IL-13 [31,32], as well as reducing cytokine levels *in vivo* [33,34]. The effects of PDE4 inhibition in diverse cell types are summarized in Table 2.

Contradictory evidence exists on the effects of PDE4 inhibition on cytokine production. In Th1-driven disease states, the ability of PDE4 inhibitors to reduce cytokine production is greater for the Th1 cytokines IL-2 and IFNγ than for IL-4 or IL-5, indicating a greater clinical benefit of Th1 cytokines in autoimmunity [35,36]. However, in allergic disease models the inhibition of Th2 cytokines such as IL-5 was greater than that for Th1 cytokines such as IL-2, GM-CSF and IFNγ, indicating a preferential Th2 inhibitory potential [37]. The basis for sensitivity to inhibition of Th1 versus Th2 cytokines thus seems dependent on the disease model. Whether PDE4 inhibitors have increased therapeutic potential in typical Th1- (eg, rheumatoid arthritis [RA] and inflammatory bowel disease [IBD]) or Th2- (eg, asthma and atopic dermatitis) mediated diseases is still unclear.

PDE4 inhibitors in asthma and COPD

Over the last 15 years there has been much excitement in the suggestion that targeting PDE4 with small-molecule inhibitors could lead to the development of novel, steroid-sparing compounds with utility in a multitude of diseases associated with chronic inflammation, particularly asthma and COPD. Several candidate PDE4 inhibitors have been investigated, but only cilomilast (GlaxoSmithkline plc) and roflumilast (ALTANA Pharma AG/Tanabe Seiyaku Co Ltd) have proceeded to pre-registration and phase III clinical trials, respectively [38,39].

Table 2. Effects of PDE4 inhibitors on immune cells.

Cell type	Broad function	Effects of PDE4 inhibitors
Mast cells	Inflammation, mediator release	Recruitment/activation, degranulation
Eosinophils	Chemotaxis, mediator release	Superoxide generation, chemotaxis, degranulation, leukotriene C_4 synthesis
Macrophages	Mediator release	Leukotriene B_4 synthesis, TNFα release
Neutrophils	Chemotaxis, mediator release	Superoxide generation, infiltration
T-lymphocytes	Proliferation, cytokine release and polarization	Proliferation/cytokine inhibition
B-lymphocytes	Differentiation, Ig secretion	IgE production
Airway smooth muscle cells	Structural remodeling	Bronchoconstriction
Epithelial cells	Structural remodeling	Bronchoconstriction

Despite encouraging data from phase III trials of these compounds in COPD, their approval has been hampered by their low therapeutic ratio. Currently, GlaxoSmithKline lists cilomilast as 'approvable' [40] and ALTANA Pharma/Tanabe Seiyaku are still investigating roflumilast in Japan [41]. There are a number of excellent reviews on the potential of PDE4 inhibitors for the treatment of asthma and COPD [42••,43••,44••,45••,46••,47], and thus they will not be discussed further in this review.

PDE4 inhibition in arthritis

Arthritis is the leading cause of physical disability worldwide and comprises over 100 different disease conditions, including RA, osteoarthritis, psoriatic arthritis, fibromyalgia and gout. RA is a chronic systemic autoimmune inflammatory disease characterized by nonspecific inflammation of the peripheral joints, potentially resulting in progressive destruction of articular and periarticular structures. Lymphocyte infiltration to the synovial tissue, as well as that of macrophages and their cytokines (eg, TNFα and GM-CSF), and increased expression of adhesion molecules, contribute to inflammatory cell emigration and retention in the synovial tissue, and disease pathogenesis [48,49].

Data from knockout mice demonstrated that TNFα and IL-1β are prominent in the pathogenesis of RA [50,51]. Genetically altered mice overexpressing TNFα spontaneously develop RA-like lesions in the joints with progressive inflammation and bone destruction [52,53]. However, disease development is abrogated by passive immunization with an anti-IL-1β receptor antibody suggesting an indirect role for TNFα [54]. Genetically susceptible DBA/1J mice show increased TNFα expression in the synovial lining of the inflamed joints following immunization with type II collagen, concurrent with the onset of symptoms [55]. It is unclear whether TNFα directly mediates this process and/or is a secondary effect linked to the expression of TNF-induced proinflammatory cytokines such as IL-1β and other chemokines [56,57]. Disease development may therefore result from the combined effect of dysregulated expression of several proinflammatory cytokines, such as TNFα and IL-1β, along with insufficient production of anti-inflammatory cytokines, such as IL-10.

The cAMP pathway represents a useful therapeutic target in RA due to its opposing effects on both TNFα and IL-10. Elevation of intracellular levels of cAMP in leukocytes by PDE4 inhibitors is accompanied by a significant inhibition of TNFα production at a transcriptional and translational level. Forskolin, an adenylate cyclase activator, also inhibits TNFα production. cAMP response element-binding (CREB) proteins are involved in synovial cell activity in patients with RA and inhibition of CREB activity corrects aberrant synovial cell functions in these patients [58]. Rolipram, a PDE inhibitor, ameliorates collagen-induced arthritis in murine experimental models [59]. Piclamilast and roflumilast, both selective PDE4 inhibitors, partially attenuate the IL-1β-induced nitric oxide production that contributes to cartilage degradation in osteoarthritis, suggesting that PDE4 inhibitors may also have chondroprotective effects [60]. While roflumilast is still undergoing clinical studies, the development of rolipram and piclamilast was discontinued in 2000 and 1996, respectively [61-63].

PDE4 activity is associated with a wide variety of inflammatory diseases and thus the regulation of proinflammatory cytokine production through PDE4 activity is being investigated for therapeutic utility in patients with RA. However, it must be noted that animal models of arthritis are induced and do not occur naturally; therefore, while they may demonstrate 'arthritis-like symptoms', the series of events are not truly reflective of human pathogenesis. For example, the collagen-induced rodent model for arthritis is based on the development of antibodies against collagen, a feature not observed in most clinical cases. This presents a serious limitation in understanding the disease-system dynamics and also the assessment of drugs and devising strategies for chemotherapeutic intervention. As a result, while preclinical data often support the clinical application of PDE4 inhibitors, the translation of this efficacy to success in trials can prove difficult.

Pathogenesis in IBD

Chronic IBDs are debilitating disorders of unknown etiology whose incidence is on the rise worldwide. Two main categories of IBD are ulcerative colitis and Crohn's disease [64,65]. Ulcerative colitis is characterized by recurring episodes of inflammation restricted to the mucosal layer of the rectum and may extend proximally. Crohn's disease is characterized by transmural inflammation of any part of the bowel, from the mouth to the anus. The prevalence of ulcerative colitis and Crohn's disease ranges from 10 to 70 per 100,000 individuals.

A variety of cytokines, such as TNFα, IL-1β, IL-6 and IL-8, contribute to immune stimulation, and cause intestinal tissue damage and T-helper (Th) cell differentiation. Th1 cells are associated principally with Crohn's disease, whereas Th2 cells are associated principally with ulcerative colitis. Existing therapies predominantly include aminosalicylates and steroids for both diseases; however, they are not useful for maintaining remission [66]. Current IBD therapy therefore utilizes nonspecific anti-inflammatory and immunosuppressive agents that are only partially effective and have serious side effects. Furthermore, a significant proportion of patients are either steroid resistant (~15 to 20%) or steroid dependent (~25 to 30%). Anti-TNFα has been explored as a therapy for IBDs, and infliximab (Remicade) became the first biological agent to be approved by the FDA for the treatment of IBD, and has since demonstrated efficacy for the acute management of inflammatory and fistulous Crohn's disease [67]. A PEGylated form of anti-TNFα antibody, CDP-870 (UCB SA, formerly Celltech), has demonstrated efficacy and is currently pre-registered in the US and Europe for Crohn's disease [68,69]. These developments have provided a rationale for the development of other biological agents that target the inflammatory cytokine pathway of the immune response.

The success of infliximab against Crohn's disease demonstrates TNFα as a functional therapeutic target. PDE4 inhibitors are potent suppressors of proinflammatory cytokines, particularly TNFα, suggesting that they could also be effective in IBD. PDE4 inhibitors stimulate the release of anti-inflammatory mediators such as IL-10 and PGE_2, which are major regulators of inflammation in the gut [70]. For example, cilomilast inhibits fibroblast-mediated collagen-gel degradation induced by TNFα and neutrophil elastase by suppressing the release of the matrix metalloproteases (MMPs) 1 and 9 [71]. Given that MMPs are associated with tissue destruction, this pathway may be an additional mode through which PDE4 inhibitors contribute to IBD therapies. PDE4 inhibitors also prevent and reverse gastrointestinal inflammation in several animal models. Their clinical benefit in respiratory disease is partly attributable to its modulation of airway remodeling. Tissue remodeling is an important feature of IBD and when extended to the gut epithelium could also translate to a therapeutic benefit in inflammation-based disorders of the gastrointestinal tract. Furthermore, the ability of PDE4 inhibitors to reduce the proinflammatory properties of leukocytes and maintain mucosal blood flow provides a spectrum of biological activities not observed with current medications. Tetomilast (Otsuka Pharmaceutical Co Ltd) is a small-molecule inhibitor of PDE4 that has shown promise in the treatment of ulcerative colitis, and is currently undergoing phase III clinical trials for this indication [72]. CC-1088, a thalidomide analog and PDE4 inhibitor, had completed a phase I/II double-blind, placebo-controlled trial in moderate-to-severe Crohn's disease which showed the compound to be safe and well tolerated [73]. However, this compound is no longer being developed by Celgene Corp, and the company is now focusing on another compound in the original series, CC-10004, for the treatment of inflammatory diseases (particularly psoriasis) [74]. PDE4 inhibition thus appears to be a viable therapeutic target for gut inflammation [70]. In addition, compounds that are either not absorbed into portal circulation or that undergo extensive metabolism (such as that indicated for budesonide) may have an improved safety profile over current therapies.

Inflammatory disorders of the skin

Psoriasis is a chronic inflammatory skin disorder caused by inflammatory cell infiltration into the dermis and epidermis, and is accompanied with keratinocyte hyperproliferation [75]. It is a complex disease and a combination of genetic and environmental factors are likely to be causative. Cytokines such as TNFα, IFNγ and chemokines orchestrate the development of psoriasis. TNFα is one of the primary cytokines that facilitates T-cell:antigen-presenting cell interaction by enhancing adhesion molecules such as ICAM-1 and thereby initiating the cascade of immune activation [76]. As PDE4 is also expressed in keratinocytes, these cells may be additional pharmacological targets of PDE4 inhibitors for the control of inflammatory skin disorders [77-80]. In fact, GlaxoSmithKline (under license from elbion AG) is developing a topical formulation of AWD-12-281 for the treatment of atopic dermatitis [81].

In addition to the indications mentioned above, PDE4 inhibitors have also been investigated for the treatment of age-related memory impairment and Alzheimer's disease [82], as well as skeletal muscle atrophy [83], hypertension [84] and autoimmune diseases [85]. However, their therapeutic benefits in these indications have yet to be demonstrated in the clinical setting.

Strategies to avoid the side effects of oral PDE4 inhibitors

The current PDE4 inhibitors in advanced stages of clinical development, cilomilast and roflumilast, have demonstrated encouraging efficacy in patients with COPD and have a superior safety/tolerability profile compared with nonselective PDE inhibitors such as theophylline [11] and first generation PDE4 inhibitors such as rolipram [14••]. The occurrence of side effects such as nausea, vomiting and gastric acid secretion has led to the search for new inhibitors with better efficacy profiles. Table 3 summarizes the current status of PDE4 inhibitors in various stages of development.

Table 3. Selected PDE4 inhibitors in development.

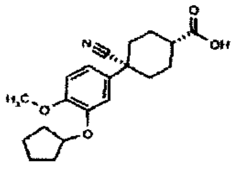
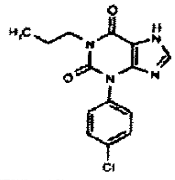
Compound	Development status	Indication
Cilomilast (GlaxoSmithKline plc) 	Pre-registration	COPD, asthma
Arofylline (Almirall Prodesfarma SA) 	Phase III trials	COPD, asthma, bronchitis

Table 3. Selected PDE4 inhibitors in development (*continued*).

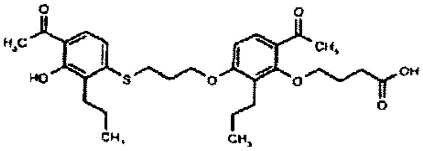
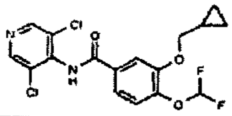
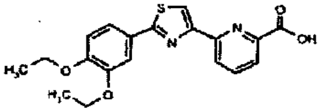
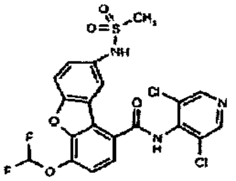
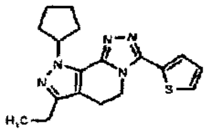
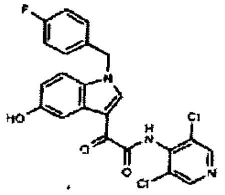
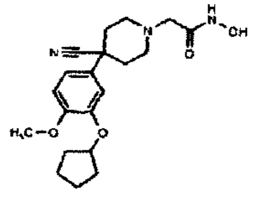
Compound	Development status	Indication
MN-001 (MediciNova Inc)  O H₃C HO S O O CH₃ OH O CH₃ CH₃	Phase III trials	Asthma, cystitis
Roflumilast (ALTANA Pharma AG/Tanabe Seiyaku Co Ltd) 	Phase III trials	COPD, asthma
Tetomilast (Otsuka Pharmaceutical Co Ltd) 	Phase III trials	COPD, IBD, ulcerative colitis
256066 (GlaxoSmithKline plc)	Phase II trials	Allergic rhinitis, COPD, inflammation, asthma
C-3885 (Merck & Co Inc)	Phase II trials	Allergic rhinitis
CC-10004 (Celgene Corp)	Phase II trials	Inflammation, psoriasis, asthma
IPL-455903 (Helicon Therapeutics Inc)	Phase II trials	Cognition disorder
MEM-1414 (Memory Pharmaceuticals Corp)	Phase II trials	Alzheimer's disease
Oglemilast (Glenmark Pharmaceuticals Ltd/Forest Laboratories Inc/Teijin Ltd)  O O=S–CH₃ NH Cl N O O N H Cl F F O	Phase II trials	COPD, rheumatoid arthritis, asthma
Tofimilast (Pfizer Inc) 	Phase I/II trials	Allergy, respiratory disease
AWD-12-281 (GlaxoSmithKline plc) 	Phase I trials	Atopic dermatitis
EHT-0202 (ExonHit Therapeutics SA)	Phase I trials	Alzheimers disease, neurodegenerative disease
LAS-37779 (Almirall Prodesfarma SA)	Phase I trials	Psoriasis
ND-125 (Evotec AG, formerly Neuro3d SA)	Phase I trials	Major depressive disorder
A series of selective cytokine inhibitory drugs, including CC-11050 (Celgene Corp)	Discovery	Congestive heart failure, inflammation, autoimmune disease, cancer, respiratory disease

Table 3. Selected PDE4 inhibitors in development (*continued*).

Compound	Development status	Indication
ONO-6126 (Santen Pharmaceutical Co Ltd) 	Discovery	Allergy, conjunctivitis
CC-7085 (Celgene Corp)	No development reported	Inflammation
KW-4490 (Kyowa Hakko Kogyo Co Ltd)	Discontinued	Asthma

The therapeutic promise of PDE4 inhibitors has been tempered due to the development of side effects, particularly nausea, emesis and headache, and potentially fatal conditions such as vasculitis and colitis [86]. All PDE4 inhibitors are emetic to a certain degree, suggesting that cAMP elevation in the emetic centers triggers an emetic response [87,88].

A correlation of low-affinity rolipram-binding site (LARBS) with inhibition of cAMP hydrolysis is related to the anti-inflammatory properties, while the high-affinity rolipram binding site (HARBS) correlates with the production of certain side effects [89]. Rolipram binds with high affinity to HARBS and low affinity to LARBS and consequently is highly emetic at therapeutic doses [90]. Second-generation PDE4 inhibitors such as cilomilast and roflumilast, however, have comparable affinities to HARBS and LARBS and hence an improved therapeutic window. Thus, drug development is faced with the challenge of alleviating the side-effect profile of PDE4 inhibitors by widening the window between efficacy and associated adversities. Identification of distinct pharmacological PDE4 forms may demonstrate improved therapeutic consequences, as it may be possible to synthesize potent inhibitors of PDE4 with low affinity for HARBS, which should, theoretically, be less emetic. However, this theory has been questioned by some investigators who feel it is too simplistic and should no longer be exploited in compound development [44••].

Another strategy involves the development of subtype-specific PDE4 inhibitors. PDE4 has four subtypes: A, B, C and D. PDE4B is the predominant PDE4 subtype in monocytes and neutrophils crucial in inflammation responses [91,92•]. PDE4D has a key role in balancing relaxation and contraction in airway smooth muscle for maintenance of airway tone [93•]. Both PDE4B and PDE4D also have complementary, non-redundant roles in the control of neutrophil function [94]. Experiments with mice deficient in the *PDE4B* or *PDE4D* gene demonstrate that emesis is linked to the selective inhibition of PDE4D [95•]. PDE4D3 has also been implicated in cardiac malfunction [96]. PDE4B$^{-/-}$ mice exhibit a profound attenuation of LPS-induced-TNFα release, which provides further rationale for exploring pharmacological inhibition of the enzyme for anti-inflammatory effects [92•]. Thus, the development of PDE4B isoform-specific inhibitors could alleviate the side effects triggered by nonspecific inhibition of PDE4D activity and thereby improve therapeutic advantage in inflammatory diseases.

Alternate possibilities to overcoming dose-limiting side effects associated with PDE4 inhibition are currently being explored. Localizing drug administration to the site of inflammation, as with the intratracheal/intranasal route of delivery for the treatment of respiratory disorders, is not only advantageous for molecules with low bioavailability, but may also overcome overt side effects. Novel dual-action based approaches combining the benefits of related therapeutic targets could also lower the effective doses of PDE4 inhibitors required to offer clinical benefit and thereby dispel unwanted systemic effects. PDE7, another cAMP-specific PDE enzyme, has also been implicated in inflammation and is present in many proinflammatory and immune cells [97]. In addition, PDE1C promotes smooth-muscle cell proliferation in arterial tissue [98]. Inhibitors which target both PDE4 and PDE7 have demonstrated a broad anti-inflammatory effect in both *in vitro* and animal models of inflammation [99,100]. Dual-specificity inhibitors which target PDE4 and PDE1C could retard smooth-muscle cell proliferation and affect airway remodeling which may provide an improved therapeutic index and better safety profile [101••].

Conclusions

Experimental evidence implicates the role of PDE4 in airway diseases, particularly the PDE4B subtype [92•]. The diversity in the regulation of cAMP and cGMP levels may be important for various other clinical conditions, which may thus benefit from pharmacological inhibition of PDE4. Preclinical data suggest that PDE4 inhibitors could be of clinical benefit to diverse diseases such as ulcerative colitis, arthritis and neuronal inflammation. The protective effects of tetomilast have been demonstrated in phase III clinical trials for ulcerative colitis as well as phase II clinical trials for COPD [82], and roflumilast [39] and rolipram [55] have shown efficacy in animal models of induced arthritis. Notably, the clinical efficacy of PDE4 inhibition is more concrete for Th2-mediated respiratory disorders and whether this will also benefit patients with Th1-mediated disorders remains to be established; however, preclinical evidence suggests that this might well be the case. PDE4 inhibition still requires further clinical investigation, especially when considering the possible side effect profiles associated with systemic exposure. Pharmaceutical companies are currently exploring the clinical benefit of a variety of PDE4 inhibitors and the results of these investigations should reveal the feasibility of these compounds as a 'pan-anti-inflammatory' therapeutics.

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Exhibit E

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Therapeutic potential of phosphodiesterase type 4 inhibition in chronic autoimmune demyelinating disease

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Abstract

It was recently demonstrated that selective phosphodiesterase type 4 (PDE4) inhibition suppresses the clinical manifestations of acute experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), and inhibits the production of tumor necrosis factor-α (TNF-α), a pathogenetically central cytokine. Since the most common presentation of MS in humans is a relapsing–remitting course, we investigated the therapeutic potential of PDE4 inhibition in the relapsing–remitting EAE model of the SJL mouse. Administration of rolipram, the prototypic PDE4 inhibitor, reduced the clinical signs of EAE during both the initial episode of disease and subsequent relapses. In parallel, there was marked reduction of demyelination and also less inflammation throughout the central nervous system (CNS) of rolipram-treated animals. Gene expression of proinflammatory cytokines in the CNS was reduced in most of the rolipram-treated animals. Additional experiments demonstrated that PDE4 inhibition acted principally by inhibiting the secretion of Th1 cytokines, however, the encephalitogenic potential of myelin basic protein-specific T cells was not impaired. Our findings suggest that PDE4 inhibitors are a promising cytokine-directed therapy in chronic demyelinating disease. © 1997 Elsevier Science B.V.

Keywords: Cytokine; Experimental autoimmune encephalomyelitis; Multiple sclerosis; Phosphodiesterase type 4; Rolipram; Tumor necrosis factor

1. Introduction

In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), a number of highly specific therapies have been developed, based on the central role of the trimolecular complex of autoreactive T cell, autoantigenic peptides and the appropriate MHC-product (Martin et al., 1992a; Wekerle et al., 1994). Interference with each partner of this complex, such as the blocking of antigen receptors on autoreactive T cells with specific antibodies, leads to a marked suppression of disease activity in inbred rodent models (Acha-Orbea et al., 1989). However, the T cell response in a genetically heterogeneous human population shows a much greater variety and complexity (Martin et al., 1992b; Giegerich et al., 1992; Utz et al., 1994). At present, the applicability of such immunotherapies to human disease remains uncertain.

Promising new treatment approaches for T cell-mediated autoimmune diseases are directed against proinflammatory cytokines. This is based on growing experimental evidence that tumor necrosis factor-α (TNF-α), the closely related lymphotoxin-α (LT), and interferon-γ (IFN-γ) are involved in the development of inflammation and tissue damage (Selmaj and Raine, 1988; Vassalli, 1992; Renno et al., 1995). The treatment of EAE and other autoimmune models with anti-TNF/LT antibodies or soluble TNF-receptors has been shown to be remarkably effective (Ruddle et al., 1990; Selmaj et al., 1995). Furthermore, we and others showed recently that substances such as rolipram, a selective cyclic nucleotide phosphodiesterase type 4 (PDE4) inhibitor, pentoxifylline, a nonspecific PDE inhibitor, or phosphatidylserine, a natural membrane phospholipid, are able to suppress TNF-production in vitro and

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EAE in vivo (Sommer et al., 1995; Genain et al., 1995; Rott et al., 1993; Monastra et al., 1993). Targeting PDE4 seems particularly promising, because this isozyme is predominantly, though not exclusively, present in inflammatory cells. The potential clinical impact of this approach could be enormous, because it targets a central pathogenic process that by-passes complex antigen receptor-specific immunoregulatory mechanisms.

Our prior study in the Lewis rat left a number of questions unanswered (Sommer et al., 1995). First, in that model, EAE is characterized by a single episode of disease, unlike the relapsing and remitting course seen in the SJL mouse EAE and in human MS; therefore the effects of rolipram on chronic disease could not be assessed. Second, the SJL mouse model of EAE has much more demyelination upon histologic examination compared with EAE in the Lewis rat, which again is more reminiscent of MS (Martin et al., 1992a). In this study, we show that long-term PDE4 suppression ameliorates clinical signs and pathological changes in chronic relapsing EAE in SJL mice, although autoreactive T cells are not eliminated from the rolipram-treated mice.

2. Materials and methods

2.1. Reagents

Rolipram: racemate and enantiomers were kindly provided by Dr. Helmut Wachtel, Schering AG, Berlin. For in vitro studies, rolipram was dissolved in a 0.25% solution of dimethylsulfoxide:saline (1:10) and further diluted in culture medium. For treatment studies, rolipram racemate was dissolved in saline and injected intraperitoneally (i.p.).

Myelin basic protein (MBP) was prepared from guinea pig spinal cords (Rockland, Gilbertsville, PA) as previously described (Deibler et al., 1972).

2.2. Mice

Female SJL/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were 8–12 weeks old when used for experiments. All procedures were in compliance with guidelines set by the NIH Animal Care and Use Committee.

2.3. Induction of EAE

EAE was induced according to previously published protocols (Racke et al., 1991). Briefly, mice were immunized with 400 μg MBP in complete Freund's adjuvant. Ten days later, MBP-specific lymph node cells (LNC) were cultured at 4×10^6 cells per ml in RPMI-1640 containing 10% FCS, nonessential amino acids, HEPES, 2-mercaptoethanol, glutamine, antibiotics, plus 25 μg/ml MBP for 4 d. Cells were then washed and injected at 3×10^7 in 0.2 ml PBS per mouse i.v. For serial adoptive transfer experiments, spleen cell suspensions were cultured and transferred in the same way.

2.4. Treatment schedules and clinical rating

Mice were treated with 1 × 6.25 mg/kg (experiment 1) or 3 × 6.25 mg/kg (experiment 2) per day rolipram (racemate) i.p. in 0.2 ml saline from day 2 after cell transfer until the end of the observation period. Control animals were injected with saline alone. Clinical signs of EAE were ranked on a scale from 0 (healthy) to 5 (tetraparetic) (Racke et al., 1994). A relapse was noted when the clinical score worsened by at least one point for at least two consecutive days.

2.5. Gene expression

After perfusion with saline, brain and spinal cord were removed and RNA extracted from whole CNS tissue using RNAzol (Cinna/Biotecs, Friendswood, TX). RNA was purified and the coupled reverse transcriptase (RT)–PCR performed as described (Svetic et al., 1991; Racke et al., 1994). Briefly, RNA samples were reverse transcribed with Superscript RT (BRL, Rockville, MD) and cytokine-specific primers were used to amplify selected cytokines. For each gene product, the optimum number of cycles was determined experimentally. To verify that equal amounts of RNA were added in each RT-PCR reaction, primers for hypoxanthine phosphoribosyl transferase (HPRT) were used in each experiment. Amplified PCR products were detected by Southern blot analysis.

2.6. Neuropathology

Animals were perfused with phosphate-buffered 2.5% glutaraldehyde as described (Racke et al., 1991). Briefly, CNS tissue was removed and thin slices were made of all levels of the neuroaxis. These were post-fixed in cold 1% osmium tetroxide for 1 h, dehydrated in ethyl alcohol and embedded in epoxy resin (Epon 812). One micrometer epoxy sections of tissue were stained with toluidine blue and examined by light microscopy in a blinded fashion.

2.7. Cell culture

Proliferative responses from LNC and spleen cells were performed by incubating 8×10^5 cells/well in triplicate or quadruplicate with or without MBP (12.5–100 μg/ml, maximum proliferation usually at 25 μg/ml) or Con A (2 μg/ml) in 96 well flat-bottom microtiter plates (Costar, Cambridge, MA) at 37°C with 5% CO_2. Cells were pulsed with 1 μCi methyl-^{3}H[thymidine] (Amersham, Arlington Heights, IL) per well for the last 16 h of the culture period and harvested on fiber filters (after a total incubation time of 96 h, if not stated otherwise). Incorporated radioactivity was measured on a Betaplate counter (Wallac, Gaithers-

Table 1
Summary of clinical data [a]

	First attack (d 7–21)		Relapse (d 22 and later)	
	incidence of EAE	MMS (± sd)	incidence of relapse	MMS (± sd)
Experiment 1 (treatment from day 2):				
Control	16/16 (100%)	3.69 (±0.58)	13/13 (100%)	3.46 (±1.01)
Rolipram (1 × 6.25 mg/kg)	12/20 (60%)	2.05 (±1.63) [b]	15/17 (88%)	2.59 (±1.09) [c]
Experiment 2 (treatment from day 2):				
Control	11/13 (85%)	2.62 (±1.27)	6/9 (67%)	1.67 (±0.94)
Rolipram (3 × 6.25 mg/kg)	3/13 (23%)	0.46 (±0.93) [b]	1/9 (11%)	0.44 (±0.96) [c]
Experiment 3 (treatment from day 17):				
Control	6/6 (100%)	3.08 (±0.45)	6/6 (100%)	2.75 (±0.62)
Rolipram (1 × 6.25 mg/kg)	6/6 (100%)	3.08 (±0.34)	4/6 (67%)	2.00 (±0.41) [b]

[a] Three representative treatment experiments are shown. Note that treatment was started on day 2 after induction in experiments 1 and 2, but only on day 17 (after peak of the first attack) in experiment 3. Animals were treated until day 58 (experiment 1), 38 (experiment 2), or 62 (experiment 3), respectively. Abbreviation: MMS mean maximum score.

[b] $p < 0.05$.

[c] $p < 0.001$.

burg, MD). For cytokine determination 8×10^6 lymph node cells were cultured in 24 well plates at 2 ml per well with or without 25 μg/ml MBP. After 12, 24, 48 or 96 h, cell free supernatants were frozen at −70°C until use. Determination of TNF-α and IFN-γ was performed by commercial ELISA (Genzyme, Cambridge, MA). Proliferation of the CTLL-2 cell line was used to detect IL-2 (Bottomly et al., 1991). IL-4 was determined using the IL-4 dependent CT.4S cell line (Seder et al., 1992) and was found to be below detection limits throughout. TGF-β was measured by a CCL-64 bioassay (Danielpour et al., 1989).

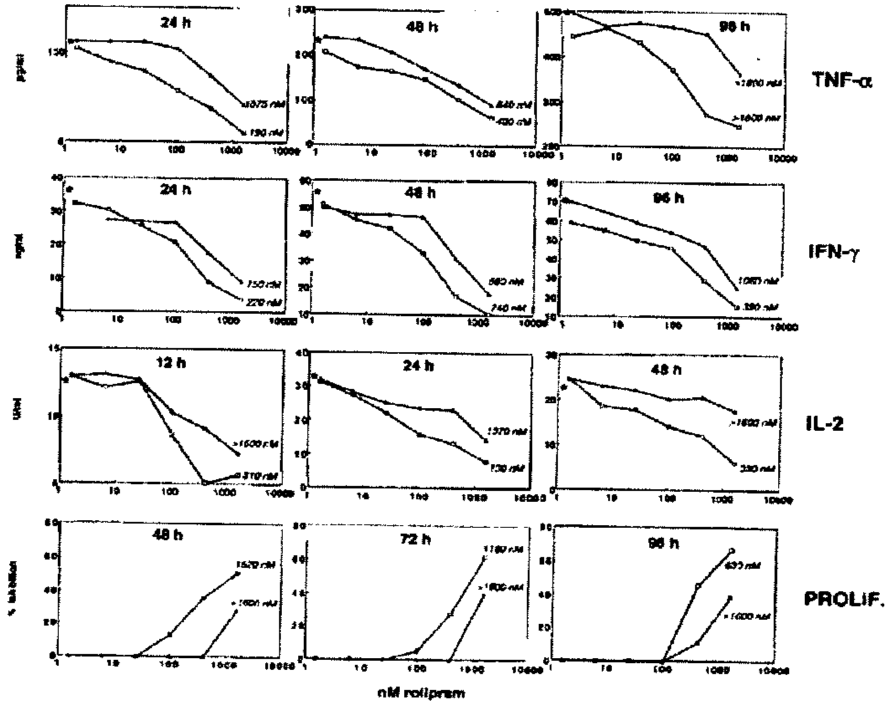

Fig. 1. Rolipram stereospecifically suppresses production of Th1 cytokines by encephalitogenic lymph node cells in tissue culture supernatants. MBP-specific LNC were cultured with MBP (25 μg/ml) and the given concentrations of rolipram: open squares = (−)-rolipram, open triangles = (+)-rolipram. Asterisks indicate the cytokine production without rolipram. Numbers in italics give the respective IC_{50} value.

3. Results

3.1. *Effect of rolipram on MBP-primed lymph node cells in vitro*

Using two enantiomers of rolipram, the production of the Th1 cytokines, IL-2, IFN-γ, and TNF-α was suppressed in a stereoselective manner, with (−)-rolipram being more potent (Fig. 1). In addition, IL-4 and TGF-β were also examined in the cell culture supernatant of the MBP-specific LNC, but were only detected in negligible amounts and unchanged by rolipram (not shown). A much higher concentration of rolipram was required to inhibit T cell proliferation than Th1 cytokines. Fifty percent inhibition of T cell proliferation by the more potent (−)-enantiomer required 600–1600 nM, whereas for Th1 cytokines this was achieved by 100–400 nM (Fig. 1). Viability of LNC, tested by trypan blue exclusion, was not impaired at concentrations of 400 nM or less or (−)-rolipram.

3.2. *Effect of in vivo administration of rolipram on chronic EAE*

Administration of rolipram following transfer of MBP-primed lymphocytes suppressed the clinical signs of chronic-relapsing EAE (Fig. 2, Table 1). Injections beginning two days after the adoptive transfer of encephalitogenic T cells resulted in lower incidence and significantly reduced severity of clinical disease in a dose-dependent manner. This effect was apparent during the first episode of disease and also during subsequent relapses.

However, it was important to observe whether rolipram would have a treatment effect, if administered after the signs of EAE were already established. In experiment 3 (Fig. 2c, Table 1) 12 mice were randomized into two groups after the initial attack and treated from day 17 post transfer. With this regimen the mean maximum score of the rolipram treated group was also reduced significantly.

3.3. *Histopathologic evaluation of rolipram treated mice*

To examine the effects of rolipram on histopathology in EAE, particularly with regard to its effect on demyelination, representative mice from experiment 2 (Table 1) were sacrificed on day 15 post transfer. Mice that received vehicle treatment displayed perivascular infiltrates associated with demyelination (Fig. 3a–b, Table 2). Some remyelination was also evident in the late acute phase of EAE. There was a marked reduction of demyelination and a modest reduction in cellular infiltration in the CNS of the rolipram-treated animals (Fig. 3c–d, Table 2). Secondary Wallerian degeneration was mainly found in untreated animals, but was very mild in the rolipram-treated group. Results from the rolipram- and the vehicle-treated groups are summarized in Table 2. Representative sections are shown in Fig. 3. The decrease in inflammation in the rolipram-treated mice was similar to our prior study in the Lewis rat (Sommer et al., 1995).

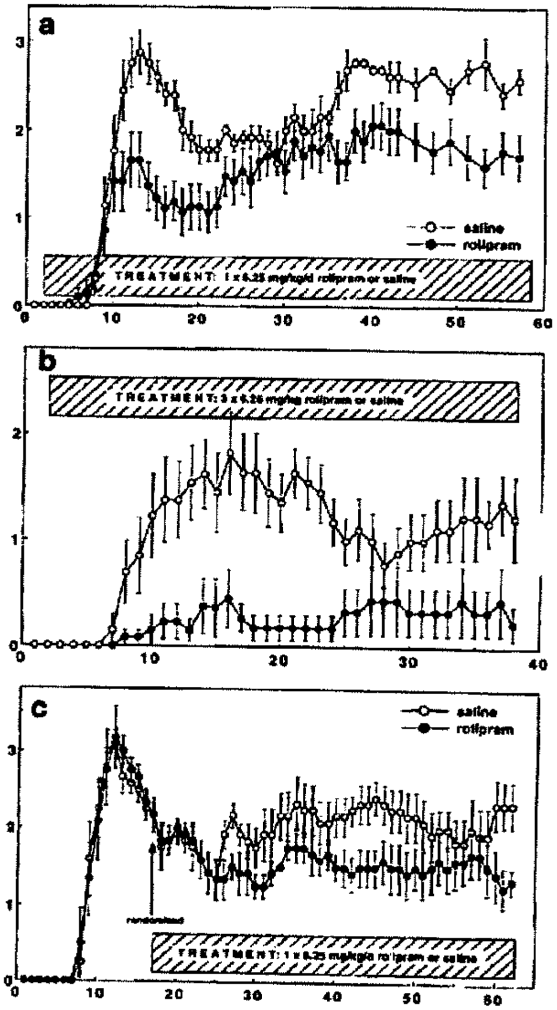

Fig. 2. Rolipram suppresses chronic-relapsing EAE. Mean clinical scores (±sem) are shown for treatment experiments 1 (a), 2 (b), and 3 (c). The hatched bars give the duration and dose of treatment. Note that in experiment 3 treatment was started during recovery from the first attack after randomization of the animals into two groups with similar clinical course. Mean maximum scores and statistical evaluation of the three experiments are shown in Table 1.

3.4. *Rolipram treatment does not deplete MBP-specific T cells*

Spleen cells from various animals in the treatment experiments were incubated in vitro for 4 d with 25 μg/ml MBP and then transferred to naive mice. Table 3 shows, that all animals, including those from the rolipram

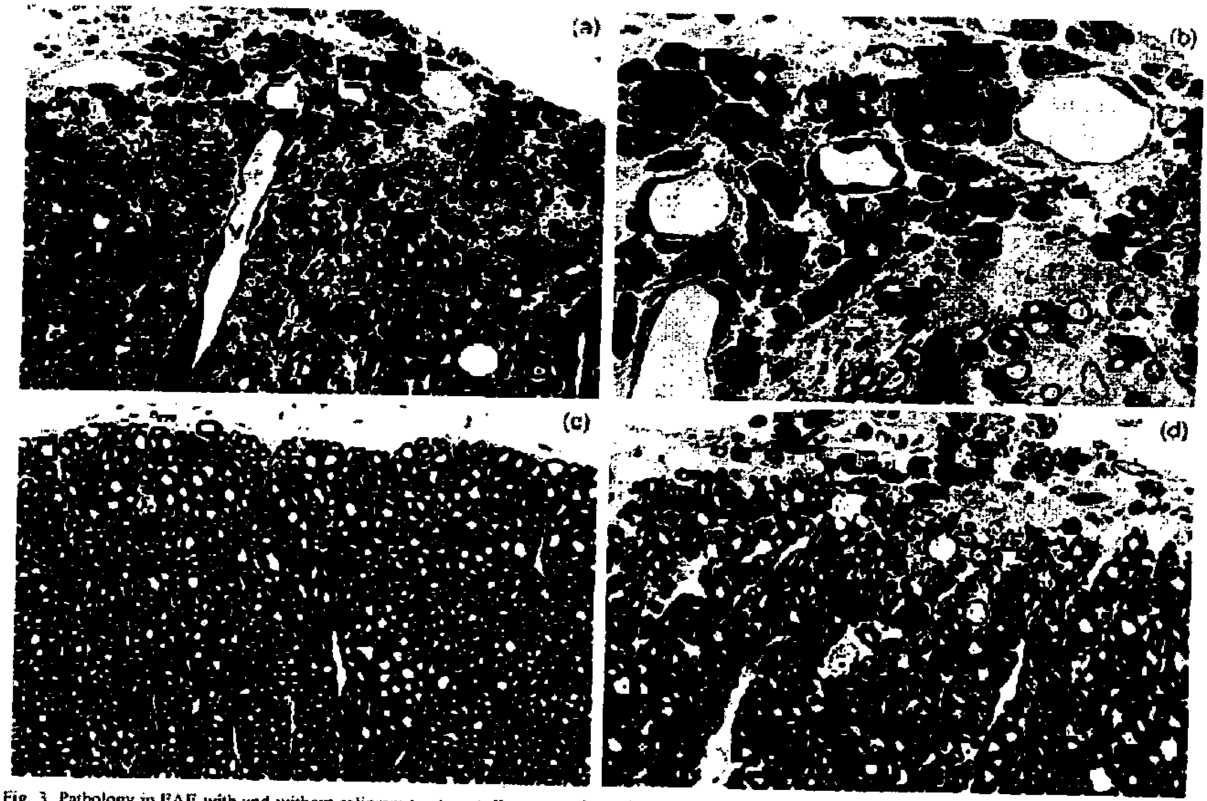

Fig. 3. Pathology in EAE with and without rolipram treatment. Representative animals from treatment experiment 2 treated with 3 daily doses of rolipram or saline (see Fig. 2, Table 1) were investigated on day 15 post transfer of MBP-specific LNC. A. Placebo treated EAE: 15 days post transfer (dpt); clinical grade 2. An area of demyelination flanks a penetrating subpial vessel (v) in an anterior column of the lumbar spinal cord. Note the perivascular and subarachnoid infiltration and the many demyelinated axons (arrows); ×350. B. Detail from A. Note the many myelin debris—laden macrophages in the leptomeningeal space and the demyelinated axons (arrows) in the underlying lesion. Some fibers display thin myelin sheaths which are probably indicative of remyelination at this late acute phase of the disease (clinical onset, 6 dpt); ×875. C. Rolipram-treated EAE; 15 dpt; clinical grade 0. In this treated animal that showed no signs of clinical EAE, the CNS parenchyma displays no changes and there is no inflammation; ×350. D. Rolipram-treated EAE; 15 dpt; clinical grade 1. A different animal from C with minimal signs of disease. Note the moderate degree of inflammation in the subarachnoid space, the small demyelinated lesions (curved arrows), the scattered myelin ovoid indicative of Wallerian degeneration (small straight arrows), and the hypertrophic astrocytes (large clear cells); ×350.

Table 2
Histopathologic analysis [a]

Treatment group	Vehicle		Rolipram	
	inflammation [b]	demyelination [c]	inflammation	demyelination
CNS location				
cerebrum/cerebellum	+ + WD	+	+ – + +	±
cervical/thoracic cord	+ + + WD	+ – + +	± – +	±
lumbar/sacral cord	+ + + WD	+ + re	±	±

[a] Rolipram treatment reduces demyelination and inflammation compared with control mice. The table summarizes CNS pathology from representative mice (two per group) in experiment 2 (treated three times daily) obtained on day 15 days after cell transfer (see Table 1, Fig. 3 for further details).

[b] Inflammation was scored according to the following system: –, no inflammatory cells; ±, a few inflammatory cells; +, organization of inflammatory infiltrates around blood vessels; + +, increasing severity of extensive perivascular cuffing with extension into the adjacent subarachnoid space.

[c] The extent of demyelination was determined according to the following scoring system: –, no demyelination; ±, a few, scattered, naked axons; +, small groups of naked axons; + +, large groups of demyelinated axons. Abbreviations: WD, Wallerian degeneration; re, remyelination.

Table 3
Summary of serial transfer experiments [a]

Donor mice			Recipient mice		
mouse	treatment	score	incidence of EAE	day of onset	max. score
day 14:					
1	rolipram	0	1/1	7	4
2	rolipram	0	2/2	7, 9	2, 3
3	vehicle	2	2/2	8, 8	3, 4
4	vehicle	2	1/3	11	2
day 28:					
1	rolipram	0	1/1	5	4
2	rolipram	4	1/1	11	5
3	vehicle	4	1/1	12	3

[a] Spleen cells from rolipram treated mice can transfer EAE as effectively as those from control mice. Spleen cell single cell suspensions from animals of various treatment groups (day 14-donor mice had been treated three times daily, day 28-donor mice once daily) were incubated with MBP (25 μg/ml) for four days and then transferred (3×10^7 cells) to naive mice to test their encephalitogenic potential. Depending on the number of spleen cells obtained from an individual donor mouse, from one to three mice could be the recipient of a serial transfer of splenocytes. The table indicates the number of mice that developed EAE from each individual donor mouse (1/3 = 3 mice received activated splenocytes, 1 developed EAE).

treatment group without any neurological deficit, could transfer EAE as effectively as control MBP-specific splenocytes. These data demonstrate that rolipram does not lead to deletion of encephalitogenic MBP-specific T cells, even after 4 weeks of continuous treatment (Table 3).

3.5. Cytokine gene expression in the CNS

Gene expression of cytokines associated with encephalitogenicity and immune regulation was examined in the CNS of rolipram-treated mice from both treatment experiments by RT-PCR. Despite considerable heterogeneity in cytokine expression in individual mice IL-2, TNF-α (except for one outlier) and IFN-γ, was decreased in most of the rolipram-treated animals (Fig. 4). IL-4 was only detected at very low levels in the animals investigated.

4. Discussion

In this paper, we extend our previous findings by showing that continuous PDE4 inhibition reduces clinical signs in a chronic–relapsing model of EAE. The clinical course and the histopathologic changes observed in the SJL mouse model of EAE used here resemble those in multiple sclerosis. Furthermore, we demonstrate that reduction of demyelination is a hallmark of PDE4 inhibition (Fig. 3, Table 2). In addition, serial transfer experiments and CNS cytokine determination in this study provide further evidence for the hypothesis that Th1 cytokine suppression by rolipram is probably important in mediating its clinical effect. Experiments in the Lewis rat model have shown that the in vivo effect of PDE4 inhibition on EAE is clearly stereospecific (N. Sommer, unpublished).

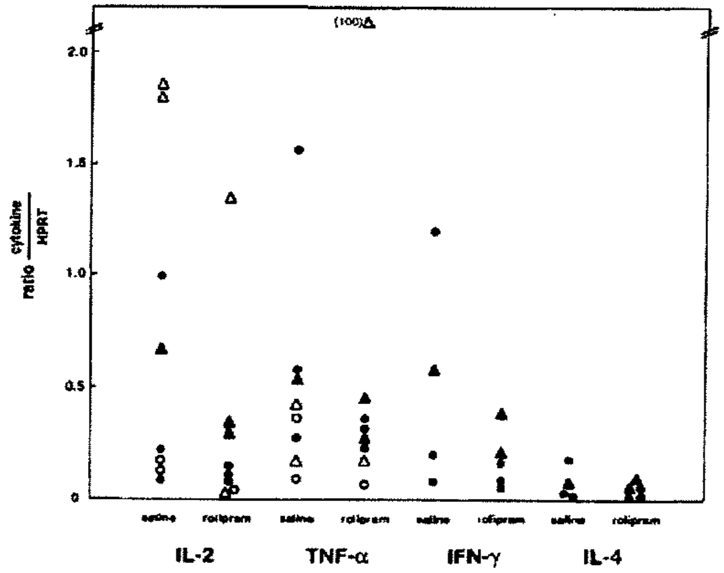

Fig. 4. Cytokine gene expression in the CNS. Cytokine gene expression was evaluated by performing Southern blots on RT-PCR cDNA from RNA isolated from the CNS of selected mice from treatment experiments 1 or 2 (treatment once daily = triangles, three times daily = circles) either on days 14–16 (closed symbols) or days 24–28 (open symbols). Values are expressed as ratio of cytokine signal divided by the constitutively expressed HPRT. In naive control mice no cytokine signal could be detected.

Based on the now well established role of TNF-α in the pathogenesis of EAE and MS, a cytokine-directed treatment approach seems reasonable and applicable to human disease (Genain et al., 1995; Sommer et al., 1995). Indeed, TNF-neutralizing treatment approaches have been followed with great effort and have reached clinical trials. For instance, in rheumatoid arthritis, anti-TNF antibody treatment using a chimerized human/mouse monoclonal antibody has already shown promising results (Maini et al., 1995). A note of caution must be made to the clinical use of macromolecules, such as monoclonal antibodies or soluble cytokine receptors. Despite their great potential, they have some typical disadvantages, namely unfavorable pharmacokinetics, parenteral administration, and high production costs. Also, long-term administration of monoclonal antibodies may not always neutralize the biologic effects of TNF. At low concentration, they may stabilize the biologically active trimeric structure of TNF and prolong its effects (Maini et al., 1995).

Therefore, novel approaches to identify low molecular weight inhibitors of cytokine production have been pursued intensively. Cyclic AMP has been known to be involved in T cell activation for some years (Wang et al., 1978; Marcoz et al., 1993). Furthermore, increasing intracellular cAMP by adenylate cyclase stimulation, phosphodiesterase inhibition, prostanoids or dibutyryl cAMP are known to have anti-inflammatory activity, including TNF-inhibition (Semmler et al., 1993; Sinha et al., 1995). Cyclic nucleotide phosphodiesterase (PDE) inhibitors are particularly interesting candidates as immunomodulating drugs, because an increasing number of even more selective and potent substances is currently being developed (Beavo et al., 1994). Among the seven PDE families the PDE4 isozymes have received the greatest attention in this respect. Inhibition of PDE4 isozymes by rolipram was their original defining property, and compared with other PDEs, they present the most complex picture concerning biochemical properties and tissue distribution. Thus far, four PDE4 genes have been identified (PDE4A/B/C/D) (Beavo et al., 1994). Rolipram is among the most potent PDE4 inhibitors described to date and has been extensively studied as long term therapy in patients with depression (Wachtel and Schneider, 1986). It has minor toxicity in humans, particularly no general immunosuppression was observed; however, some dose-limiting effects consisted of gastrointestinal symptoms, such as nausea and emesis (Zeller et al., 1984). Undoubtedly, the ongoing development of new selective PDE4 subtype inhibitors could increase tissue selectivity and clinical potency, while reducing side-effects. So far, both message and protein for PDE4A and PDE4B, but not PDE4C or PDE4D, have been shown to be up-regulated in human monocytes by activation of protein kinase (Torphy et al., 1995).

The precise molecular mechanism of rolipram action is not yet clear. Current evidence suggests that it inhibits the production of proinflammatory cytokines, best shown for TNF-α (Prabhakar et al., 1994; Genain et al., 1995; Sommer et al., 1995; and this study). Here, we have shown that gene expression of several proinflammatory cytokines is reduced in the target organ, the CNS (Fig. 4). These data must be interpreted with caution, since they depend on differences in the magnitude and cell types of the inflammatory infiltrates and do not necessarily reflect protein levels of the respective cytokines. Our data, in accord with data from a monkey model of EAE (Genain et al., 1995) and in vitro findings in human monocytes (Prabhakar et al., 1994), suggest that rolipram may act, in part, by inhibiting TNF-α and IL-2 production in vivo. Furthermore, it is now generally agreed that cell types involved in inflammatory processes contain PDE4 in relevant quantities (Torphy et al., 1992), though some inflammatory cells may also contain PDE3. Preliminary data suggest that in human MBP-specific Th1 cells PDE4 is more prevalent than PDE3 and correlates well with cellular activation processes (Ekholm et al., unpublished). This study provides further support for the concept that PDE4 inhibition may be useful as a therapy for diseases mediated by Th1 cytokines, such as multiple sclerosis.

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